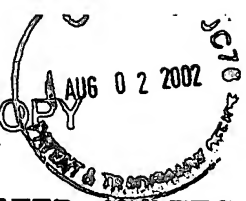


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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Conf. No.: 7383
WOLFF et al	)	Examiner:
Appln. No.: 10/092,947	)	Washington, D.C.
Filed: March 8, 2002	)	August 2, 2002
For: RECOMBINANT DIMORPHIC	)	Atty.Docket:
FUNGAL CELL	)	WOLFF=3
	)	

REQUEST FOR PRIORITY

Honorable Commissioner for Patents  
Washington, D.C. 20231

Sir:

In accordance with the provisions of 37 CFR §1.55 and the requirements of 35 U.S.C. §119, filed herewith a certified copy of:

Danish Appln. No. PA 2001 00395 Filed: 8 March 2001.

It is respectfully requested that applicant be granted the benefit of the priority date of the foreign application.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.  
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# Kongeriget Danmark

Patent application No.: PA 2001 00395  
Date of filing: 08 March 2001  
Applicants: Bioteknologisk Institut  
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This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

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**Patent- og  
Varemærkestyrelsen**  
Erhvervsministeriet

Taastrup 07 March 2002

Inge-Lise Sørensen  
Head Clerk

**Recombinant dimorphic fungal cell comprising a regulatable expression of a regulator of morphology**

**Technical Field of the Invention**

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The present invention relates to a recombinant, dimorphic fungal cell comprising a regulatable expression of a regulator of morphology. The nucleotide sequence encoding the regulator of morphology is operably linked to an expression signal not natively associated therewith. Expression of the regulator of morphology directed by the expression signal not natively associated therewith results in a dimorphic shift and/or an improved filamentation of the dimorphic fungal cell. The improved filamentation of the dimorphic fungal cell is positively correlated with an increased production and/or secretion of a desirable polypeptide.

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It is an object of the present invention to provide fungal host organisms capable of expressing recombinant proteins while at the same time exhibiting satisfactory growth characteristics. It is a further object to provide - in a single fungal host organism - the characteristic of homogeneous growth and low viscosity typically associated with yeast organisms, and the capability for high protein secretion normally associated with filamentous fungi.

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**Background of the Invention**

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The use of recombinant host cells in the expression of heterologous proteins has in recent years greatly simplified the production of large quantities of commercially valuable proteins, which otherwise are obtainable only by purification from their native sources. Currently, different expression systems including prokaryotic and eukaryotic hosts are available. The selection of an appropriate expression system will often depend on the ability of the host cell to produce adequate yields of the protein as well as on the intended end use of the protein and the requirement for post-transcriptional modifications (e.g., glycosylation, etc).

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Although mammalian cells and yeasts are the most commonly used eukaryotic hosts, filamentous fungi may possibly also be used as host cells for recombinant

protein production. However, the use of filamentous fungi for recombinant protein production is often associated with several practical problems.

Filamentous fungi are recognized for their ability to produce and secrete high amounts of proteins, and certain species of e.g. the genus *Aspergillus* have been used effectively as host cells for recombinant protein production. Although *Aspergillus niger* and *Aspergillus oryzae* are routinely used in recombinant protein production, several notable drawbacks are associated with using such strains.

In particular, the morphology of filamentous fungi is not optimal for growth in fermentors, as the viscosity of the culture tends to become rather high as biomass increases. Increased viscosity limits the ability to mix and aerate the fermentation culture, and this leads to oxygen and nutrient starvation of the mycelia, which therefore becomes non-viable and unproductive.

Furthermore, filamentous growth in a fermentor is often associated with the formation of an uneven distribution of too dense aggregates of mycelium. This also results in nutrient starvation.

Accordingly, for commercial purposes, there continues to be a need for fungal host organisms capable of expressing recombinant proteins while at the same time exhibiting satisfactory growth characteristics. In particular, there is a need for combining - in a single fungal host organism - the characteristic of homogeneous growth and low viscosity typically associated with yeast organisms, and the capability for high protein secretion normally associated with filamentous fungi.

Many members of the fungal kingdom have a distinguishing feature, dimorphism, which is the ability to switch between two morphological forms: a yeast form and a filamentous form. The morphological transition between yeast and filamentous forms is rather variable and encompasses pseudohyphal and true filamentous growth.

Some species display pseudohyphal but also true filamentation (e.g., *Candida*). Yet another class of dimorphism occurs when differentiated hyphae disarticulate to generate young cells, called arthroconidia or thalloconidia (e.g., *Geotrichum*, *Arxula*; Wartmann et al., 1995).



The morphological state of these organisms is determined by a combination of environmental stimuli and is often associated with pathogenesis (Madhani and Fink 1998). The yeast stage is normally unicellular and uninucleated, while the filamentous phase is multicellular. A remarkable exception to this rule is found in fungi  
 5 belonging to the Zygomycetes (e.g., *Mucor*). In *Mucor*, both filamentous and yeast growth is organized in a single multinucleated cell. *Mucor* yeasts are multipolar (daughter cells can originate at different positions of the mother cell as opposed to bipolar and monopolar) each harboring more than one nucleus, while mycelium is  
 10 aseptate but with evenly distributed nuclei.

Although *Mucor* species display a variety of differentiated hyphal morphologies, mainly associated with arthrospores, sporangiospores, or zygospores, only those species capable of growing in the form of spherical, multipolar, budding yeasts are  
 15 referred to as dimorphic. Examples of dimorphic *Mucor* species include *M. racemosus*, *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and certain strains of *M. subtilissimus*.

Species of *Mucor* genetically constrained to a monomorphic existence include *M. mucedo*, *M. hiemalis*, *M. miehei*, *M. pusillus*, *M. rammanianus*, and certain strains of  
 20 *M. subtilissimus*. Besides *Mucor*, only two other genera of dimorphic zygomycetes have been described. These are *Mycotypha* and *Cokeromyces*, both of which within the order Mucorales.

*Mucor* species are capable of generating three different types of spores. Zygospore  
 25 are spiny, black, thick-walled structures. Germination of zygospores result in the formation of sporangiospores. Sporangiospores are formed only on a solid substratum under an aerobic atmosphere. *Mucor* sporangiospores are characteristically ellipsoidal in shape.

30 The sporangiospore is capable of developing into either the yeast or hyphal form upon germination, the precise morphological direction taken being dependent, among other things, on the nutritional and gaseous environments. This capability establishes *Mucor* species as unique among commonly studied microbial models of  
 35 development in that they are faced with a bifurcation in the morphogenetic sequence

at which critical regulatory responses presumably direct the organism to construct one or the other of alternative morphologies. Irrespective of whether the sporangiospore ultimately develops into a budding yeast cell or a hyphal germ tube, it is initially likely to undergo a period of spherical growth.

5

Arthrospores represent the least studied and most poorly understood cell type made by *Mucor* species. Although arthrospores derive only from hyphae, arthrospores from dimorphic species of *Mucor* also have the ability to germinate into either yeasts or hyphae depending on their environment. Such morphogenetic conversions have not yet been studied or described in any detail.

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Accordingly, *Mucor* hyphae may develop from any of the spore types mentioned above and from *Mucor* yeasts, and *Mucor* yeasts may develop from sporangiospores, arthrospores, and hyphae of dimorphic species.

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The initial phase of yeast development from sporangiospores is indistinguishable from that described above for hyphal development from sporangiospores. It should be noted that hyphal fragments persist as a significant portion of the cell population for a considerable time after the initiation of hypha-to-yeast morphogenesis. The resulting, mixed-cell population has so far dissuaded researchers from studying morphological conversions in this direction in any significant detail.

20

Much of the knowledge about the molecular mechanisms underlying the dimorphic switch has been obtained from studies of yeast and pseudohyphal differentiation in *Saccharomyces cerevisiae*. Different signal transduction pathways are involved in regulating the transition between these two forms in the budding yeast *S. cerevisiae* (Roberts and Fink 1994; Fig. 5), and evidence is now emerging that homologous signaling modules are involved in regulating filament formation in a range of other fungi. In *S. cerevisiae* and *C. albicans*, parallel signal transduction pathways are involved in dimorphism, namely a cAMP dependent and a MAP kinase-dependent pathway (Fig. 5). However, large differences in the stimuli, regulation and control of the dimorphic shift are found in different fungi. As an example, exogenous addition of cAMP to *Candida albicans* (Ernst 2000) promotes filamentation whereas in *Mucor racemosus* results in 'constitutive' yeast growth (Orlowski 1991). Thus, different role and control of the cAMP dependent protein kinase A in these two fungi must exist.

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Biochemical data on *Mucor* dimorphism have been extensively reviewed (Orlowski 1991). Generally, anaerobiosis and the presence of a fermentable hexose result in yeast growth, while aerobiosis and nutrient limitation are associated with filamentous growth. However, a gradient in the requirements for yeast or filamentous growth is found within the genus *Mucor*. *M. genevensis* can grow as yeast in aerobiosis if supplied with a high concentration of hexose; *M. rouxii* requires both hexose and anaerobic conditions to grow as a yeast and *M. racemosus* cannot grow as yeast anaerobically at all unless an hexose is present in the medium.

In addition to morphopietic agents, oxygen, CO<sub>2</sub>, and hexoses occurring naturally in the external environment, a variety of synthetic compounds have also been reported to alter *Mucor* cell morphology. Some, but not all of these, have been useful when studying the regulation of *Mucor* morphogenesis.

Substances that inhibit mitochondrial energy-generating functions lock at least some *Mucor* species into the yeast form under aerobic conditions. This prevents the generation of an increased filamentation and/or prevents a dimorphic shift from yeast to filamentous fungal cell.

Also, certain morphopoietic agents with the capacity to induce aerobic growth in the yeast form do so only if a hexose is present in the medium. It has also been reported that e.g. an elevated level of fermentative metabolism can be observed in cells chemically induced to grow as yeasts under aerobic conditions. This observation suggests a linkage between alcoholic fermentation and yeast morphology, but the issue remains largely unresolved.

Although a given set of environmental parameters and conditions can generally be expected to evoke the same morphological response from sporangiospores, arthrospores, and vegetative cells on solid as well as liquid growth media, researchers studying *Mucor* species with respect to their morphological responses to the environment have in some cases found enough variation to preclude any unqualified extrapolation of experimental results from one system to another.

Intracellular cAMP concentrations may play a role in the morphology of dimorphic fungi, and yeast morphology is characterised by an intracellular level of cAMP that has been reported about 3-fold higher than the level of cAMP in filamentous cells. An immediate target of cAMP is the cAMP-dependent protein kinase A (PKA). cAMP  
5 is also known to act primarily as an effector of PKA in eukaryotic cells.

Extensive knowledge has been gained about PKA in a variety of experimental systems (for review, see Taylor et al., 1990). PKA consists of two regulatory subunits (PKAR) that bind to and inhibit the activity of two catalytic subunits (PKAC).  
10 In the presence of cAMP, PKAR dissociates from PKAC, resulting in free catalytic subunits that are the active kinase. Three major structural features are present in PKAR. A dimerization domain is located in the N-terminal one-third of the protein and has been shown to mediate dimer formation between two PKAR subunits and interaction with other proteins. The regulation of PKA activity is inversely propor-  
15 tional to the concentration of cAMP. Binding of cAMP to PKAR results in the release of PKAC subunits and the triggering of a kinase cascade resulting in morphogenesis, differentiation and dimorphism. Earlier work identified two species of cAMP-binding proteins in *M. racemosus* and *M. genevensis* with similar molecular weight (51- or 65-kD; Forte and Orlowski 1980). At least one of these protein species may  
20 represent PKAR. Remarkably, in the related fungus *M. rouxii*, PKAR was identified as a 70-kDa protein (Moreno and Passeron 1980).

The involvement of PKA in polarized growth has been shown using of cAMP analogues. Addition of these analogues mimics activation of PKAC and results in  
25 *Mucor* yeast growth under aerobic conditions. Likewise, the regulation of PKA activity is crucial throughout the filamentation phase, since the removal of analogues results in the immediate shift from isodiametric to filamentous growth (Rossi et al., 2000; Orlowski 1991). Recently, cloning of the cAMP binding domains of the *M. rouxii* PKAR and recombinant production in *E. coli* has been reported (Sorol et al.,  
30 2000).

Addition of cAMP analogues has also been shown to repress the *de novo* synthesis of MRAS3, one of the three RAS proteins found in *Mucor* (Roze et al 1999). The RAS superfamily of small GTP-binding proteins includes signal-coupling proteins  
35 which are components of an intracellular signaling network mediating an appropriate

cellular response to external stimuli. MRAS3 is mainly associated with polar growth, as well as being involved in other processes like sporulation and germination, while MRAS1 is only associated with the regulation of polar growth (Roze et al 1999). As for PKA activity, activation of a RAS-MAP kinase pathway in *S. cerevisiae* and *C. albicans* leads to high cAMP levels and pseudohyphal development, whereas in *Mucor* and in the maize pathogen *Ustilago maydis* high levels of cAMP constrain growth to the yeast form (Orlowski 1991; Borges-Walmsley and Walmsley 2000).

A few examples of heterologous protein production have been described for *Mucor racemosus*. The production in *M. racemosus* of a *Mucor miehei* aspartic protease (MmAP) represents one example of recombinant protein production in *Mucor* (Harboe et al., 1987). In this case, the native promoter and the secretion signal of MmAP were used. Attempts to produce recombinant calf chymosin in *M. racemosus* are also reported for the same expression and secretion signals, although the levels obtained were extremely low (Strøman et al., 1990). In both cases, filamentous growth on solid medium was preferred for protein production and secretion.

Other examples of recombinant proteins produced in *M. racemosus* are limited to enzymes involved in biosynthesis of amino acids and carotenes. Direct selection in an auxotrophic host (Iturriaga et al., 1992) or expression of homologous genes involved in carotenogenesis has been exploited (Ruiz-Hidalgo et al., 1999; Navarro et al., 2000). In these cases, the natively associated signals were used to express the gene of interest.

A dimorphic fungal cell, *Arxula adeninivorans*, has recently been developed for the production of recombinant proteins (Wartmann et al., 2000). The type of morphology is dependent of the growth temperature. Filamentous growth is obtained by increasing temperature above 42 °C. This approach has been proposed solely to enhance secretion of the recombinant protein. It is assumed in the art that filamentous fungi have a larger secretion capacity than yeasts, although interspecific comparisons are somewhat cumbersome.

However, the proposed use of a temperature shift is normally associated with the triggering of a heat shock response in the cell. Among the physiological and cellular effects of heat shock, the synthesis of a number of stress proteases is initiated and

maintained during the period of growth at high temperature and the degradation of heat denatured proteins is very effectively carried out.

### **Summary of the Invention**

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Although dimorphic fungi including Mucor species are capable of growing as either single yeast cells or as a filamentous fungi, the shift between growth as yeast and filamentous growth requires the activation of a complex genetic machinery that is at present not well understood. This lack of understanding has prevented dimorphic  
10 fungi from being used as efficient host organisms for e.g. heterologous gene expression.

Also, anaerobic growth is associated with sugar fermentation and production of ethanol. This process leads to strong growth inhibition. In order to overcome this  
15 problem, the controlled expression of one or more key genes involved in the control of dimorphism or filamentation is preferred.

Accordingly, there exists a need for industrially applicable, dimorphic fungi capable of shifting morphology - rapidly and controllably - from a single cell morphology to a  
20 morphology characterised by a filamentous growth. In particular, there exists a need for such cells having an improved filamentation capability resulting in an increased production and/or secretion of a desirable polypeptide.

The lack of suitable genetic tools such as strong and regulated promoters has  
25 represented a significant drawback for the utilization of Mucor for production of heterologous proteins. However, the fact that Mucor is capable of growing either as a yeast or as a filamentous fungal cell represents a technological advantage, provided the shift between the two growth types can be made based on a set of readily controllable parameters.

30

It is an object of the present invention to provide fungal host organisms capable of expressing recombinant proteins while at the same time exhibiting satisfactory growth characteristics.

It is a further object to provide - in a single fungal host organism - the characteristic of homogeneous growth and low viscosity typically associated with yeast organisms, and the capability for high protein secretion normally associated with filamentous fungi.

5

Accordingly, the present invention provides novel recombinant fungal host cells and a novel fermentation procedure in which the host strain is engineered in order to exhibit growth characteristics particularly well suited for growth in fermentors during the biomass production phase and for protein secretion during the heterologous protein production phase.

10

The host cells of the invention are capable of rapid growth as multipolar yeasts, which exhibit low viscosity even at a high biomass concentration and result in evenly dispersed cells allowing sufficient diffusion of nutrients. In particular, the host cells of the invention are dimorphic fungi, which under appropriate fermentation conditions grow as multipolar yeasts. When suitable levels of biomass are obtained, the filamentous growth is regulated by regulating the expression of at least one of a group of regulatory genes involved in the control of dimorphism and filamentation, wherein said at least one gene is operably linked to a suitable regulated promoter.

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Preferred fungal hosts produce a homogeneous yeast culture under non-induced conditions. Most preferably, the fungal host cells are selected from the group consisting of *Mucor* sp., and other dimorphic Zygomycetes. Also other dimorphic fungi where control of the dimorphic shift can be regulated during growth in fermentor are preferred.

25

The invention therefore provides recombinant fungal host cells, as defined above, comprising a nucleic acid fragment encoding a heterologous protein (which is herein understood also to encompass peptides), which protein can be expressed by the host cell.

30

The invention further provides a method for production of heterologous proteins comprising the step of culturing a host cell of the invention under conditions conducive to the expression of the heterologous protein of interest, and comprising the further step of increasing the filamentation and/or controlling the induction of

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filamentation by increasing or decreasing the expression of a regulator of filamentation and dimorphism in any appropriate genetic background, and recovering the heterologous protein from the culture, including any recovery of the protein from the supernatant of the culture.

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In one preferred aspect of the present invention, there is provided an isolated polynucleotide comprising

- 10 i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- 15 ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

wherein the first and second nucleotide sequences are not natively associated.

20 In another aspect, the present invention relates to a fungal host cell transformed or transfected with such a polynucleotide, wherein said fungal host cell optionally further comprises

- 25 i) at least one nucleotide sequence encoding a gene product, and operably linked thereto,
  - ii) at least one further nucleotide sequence comprising a further expression signal capable of directing the expression in a dimorphic fungal cell of the at least one nucleotide sequence encoding the gene product, wherein said further expression signal is regulatable, during growth of the
- 30
- a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH,



low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,

5                   b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,

                  c) the growth phase of the dimorphic fungal cell, and

10               d) the growth rate of the dimorphic fungal cell.

wherein the nucleotide sequence encoding the gene product and the further nucleotide sequence comprising the regulatable expression signal are not natively associated.

15

In a further aspect there is provided a dimorphic fungal cell comprising

                  i)       at least one nucleotide sequence encoding a gene product, and operably linked thereto,

20

                  ii)      at least one further nucleotide sequence comprising a further expression signal capable of directing the expression in a dimorphic fungal cell of the at least one nucleotide sequence encoding the gene product, wherein said further expression signal is regulatable, during growth of the  
25                   dimorphic fungal cell, by one or more of

                  a)      the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH,  
30                   low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,

                  b)      the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat  
35                   shock genes,

- c) the growth phase of the dimorphic fungal cell, and
- d) the growth rate of the dimorphic fungal cell.

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wherein the nucleotide sequence encoding the gene product and the further nucleotide sequence comprising the regulatable expression signal are not natively associated.

10 In a still further aspect, the above-mentioned dimorphic fungal cell is transfected or transformed with the polynucleotide comprising

- 15 i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

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wherein the first and second nucleotide sequences are not natively associated.

In further aspects, the present invention relates to a method for constructing a recombinant fungal cell, or a recombinant dimorphic fungal cell, said method  
25 comprising the step of transforming or transfecting a polynucleotide, or a vector comprising said polynucleotide, into a fungal cell or a dimorphic fungal cell.

The method for constructing a recombinant fungal cell, or a recombinant dimorphic fungal cell, preferably comprises the further steps of

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transforming or transfecting said recombinant fungal cell or said recombinant dimorphic fungal cell with a further polynucleotide comprising

- 35 i) at least one nucleotide sequence encoding a gene product, and operably linked thereto, and

ii) at least one further nucleotide sequence comprising a further expression signal capable of directing the expression in a dimorphic fungal cell of the at least one nucleotide sequence encoding the gene product, wherein said further expression signal is regulatable, during growth of the dimorphic fungal cell, by one or more of

a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,

b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,

c) the growth phase of the dimorphic fungal cell, and

d) the growth rate of the dimorphic fungal cell,

wherein the nucleotide sequence encoding the gene product and the further nucleotide sequence comprising the regulatable expression signal are not natively associated.

In a further aspect the present invention provides a method for regulating the morphology of a recombinant fungal cell or a recombinant dimorphic fungal cell, said method comprising the steps of

i) cultivating said fungal cell or said dimorphic fungal cell under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and

- 5                    ii)        regulating the morphology of said recombinant fungal cell or said recombinant dimorphic fungal cell, wherein said regulation of the morphology results from regulating the expression in said recombinant fungal cell, or said recombinant dimorphic fungal cell, of said regulator of morphology.

In a further aspect there is provided a method for obtaining a predetermined dimorphic shift of a dimorphic fungal cell, said method comprising the steps of

- 10                    i)        cultivating said dimorphic fungal cell under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and
- 15                    ii)        obtaining a predetermined dimorphic shift of said dimorphic fungal cell, wherein said dimorphic shift results from regulating the expression in said dimorphic cell of said regulator of morphology.

20                    In a still further aspect the present invention provides a method for increasing the filamentation of a fungal cell, or a dimorphic fungal cell, said method comprising the steps of

- 25                    i)        cultivating said fungal cell, or said dimorphic fungal cell, under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and
- ii)        increasing the filamentation of said fungal cell, or said dimorphic fungal cell, wherein said increased filamentation results from regulating the expression in said dimorphic cell of said regulator of morphology.

30                    In a still further aspect there is provided a method for increasing the secretory capacity of a fungal cell, or a dimorphic fungal cell, said method comprising the steps of

- i) cultivating said fungal cell, or said dimorphic fungal cell, under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and
  - 5 ii) increasing the secretory capacity of said fungal cell, or said dimorphic fungal cell, wherein said increased secretory capacity results from regulating the expression in said dimorphic cell of said regulator of morphology.
- 10 In an even further aspect there is provided a method for producing a gene product in a fungal cell, or a dimorphic fungal cell, said method comprising the steps of
- i) cultivating said fungal cell, or said dimorphic fungal cell, under conditions allowing expression of said first nucleotide sequence encoding said at least one regulator of morphology, and
  - 15 ii) cultivating said fungal cell, or said dimorphic fungal cell, under conditions allowing expression of said nucleotide sequence encoding said gene product, and
  - 20 iii) producing the gene product.

#### **Brief Description of the Drawings**

25 FIG.1 to FIG. 4. Following a shift from anaerobic to aerobic growth conditions, a transition of Mucor morphology gradually occurs from a unicellular, essentially spherical morphology to filamentous structures characterised by an aseptate mycelium comprising multinucleated cells.

30 FIG.1 and FIG. 2 illustrate the unicellular, essentially spherical morphology of *M. racemosus* that can be observed during anaerobic growth. Upon shift to aerobic conditions, a phase of growth in diameter is observed (panels 1 to 18), representing the first 3 h after the shift.

FIG. 2 and FIG. 3 illustrate the subsequent development of numerous protruding structures (panels 19-32) which evolve into hyphae. Hyphae are structures that show a HGU (hyphal growth unit) of more than zero (value for yeast growth). Hyphal development (i.e., elongation) occurs rapidly (panels 33-40).

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FIG. 3 and FIG. 4 illustrate that branching of hyphae becomes evident and proliferates following the first 7 h after the shift (panels 41-60).

FIG.5. Signal transduction pathways controlling filamentation and dimorphism in fungi. Two major pathways are depicted, the cAMP dependent (cAMP boxes) and the MAP kinase pathway (MAPK boxes). The gene nomenclature for each organism is used and the common denomination is shown below in brackets for comparison.

10

FIG.6. Structure and expression of the *M. racemosus* *pkar* gene. A) Overview of the *pkar* insert in the genomic library clone showing the coding region (grey boxes and arrow) and the position of the two introns (black boxes); the region included in *pkar1-3b* was used as a probe and is depicted as a thick line; relevant restriction sites are shown (N, *Nde*I; X, *Xho*I); B) Northern blot analysis using RNA obtained from R7B growing anaerobically in Vogel's medium containing 2% casaminoacids (lane 1) and the same culture 4 h after the shift to aerobic conditions, with no glucose added (lane 2), or glucose added to final concentrations of 2 percent, 5 percent, and 10 percent, respectively (lanes 3 to 5).

15

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FIG.7. Multiple alignment of PKAR. The *M. racemosus* (Mrac) PKAR was aligned with relevant fungal and human PKAR sequences using the Clustal alignment of the MEGALIGN program (DNASTar, Lasergene). Abbreviations: Mrou, *Mucor rouxii*; Beme, *Blastocadiella emersonii*; YBCY1, *Saccharomyces cerevisiae* BCY1; HKAP1, Human KAP1. Boxes show positions of residues identical to the consensus.

25

FIG.8. Overexpression of PKAR in *M. racemosus*. (A) Plasmid map of pEUKA4::*pkar*, (B) Expression and transcription start site for *pkar*. (left panel) Northern blot analysis of KFA121 grown on YNB supplemented with 5 % glucose (lane 1). The same conditions were used for the vector control strain (lane 2). The RNA gel is shown below for loading control. (Right panel) Primer extension: The fragment obtained is indicated with an arrow; a sequence ladder was run on

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pEUKA4::*pkar* to determine the transcription start site (tss). The sequence obtained is shown below (arrow, tss; mRNA sequence in italics, cloning site (*XhoI*) and ATG start codon of *pkar* (bold). (C) Colony morphology of KFA121 (right) and the vector control strain (left) on YNB plates (2 % glucose).

5

FIG.9. The *M. racemosus* STE12 homologue. A) Protein sequence homology: the *M. racemosus* (Mrac) STE12 was aligned with relevant fungal STE12 sequences using the Clustal alignment of the MEGALIGN program (DNASTar, Lasergene). Abbreviations: Scer, *S. cerevisiae* STE12; Calb, *C. albicans* CPH1; Klac, *Kluveromyces lactis* STE12; Anid, *Aspergillus nidulans* STE12; Cpur, *Claviceps purpurea* STE12.

10

Fig.10. The *M. racemosus* MAP kinase homologue. A) Overview of the gene sequence obtained (open boxes, coding region; black boxes, introns, dashed block arrow, estimated full length gene). B) The fragment of the *M. racemosus* MPK1 protein was aligned with relevant sequences using the Clustal alignment of the MEGALIGN program (DNASTar, Lasergene). Abbreviations: MPK1, *M. racemosus* MPK1 (fragment); SPM1, *S. cerevisiae* SPM1; MKC1, *C. albicans* MKC1; FMK1, *Fusarium oxysporum* FMK1; SLT2, *S. cerevisiae* SLT2; ERK1, *C. albicans* ERK1

15

Fig.11. The *M. racemosus* MAP kinase kinase kinase homologue STE20. The fragment of the deduced *M. racemosus* STE20 protein was aligned with relevant sequences using the Clustal alignment of the MEGALIGN program (DNASTar, Lasergene). Abbreviations: Mrac, *M. racemosus* STE20 (fragment); Sce, *S. cerevisiae* STE20; Spo, *S. pombe* PAK1; Cal: *C. albicans* CLA4; Mou: Mouse SPAK1. Only the first 450 aa of the database sequences were used for the alignment.

20

25

Fig. 12. Nucleotide sequence and derived amino-acid sequence of *gpd1*. Numbering of nucleotides is with respect to the start of the coding sequence. Exon sequences are capitalised. Sequences with homology to the lariat formation consensus sequence within introns are italicised. Putative TATA and CAAT boxes are boxed and bolded, respectively. Pyrimidine stretch is underlined. The putative polyadenylation signal is double underlined. The transcription start point is capitalised and bolded. The sequence corresponding to the gene-specific oligonucleotide used in Northern blotting and primer extension is wavy underlined.

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Fig. 13. Nucleotide sequence and derived amino-acid sequence of *gpd2*. For details, see legend to Fig. 12.

5 Fig. 14. Nucleotide sequence and derived amino-acid sequence of *gpd3*. For details, see legend to Fig. 12.

Fig. 15. A: Intron positions in GPD encoding genes from I: Zygomycetes; II: Basidiomycetes; and III: Ascomycetes. B: Sequence alignment of the amino acid sequences of the GPD proteins encoded by *gpd1*, *gpd2* and *gpd3*. Identical residues are boxed. C: Phylogenetic analysis of GPD sequences using the Clustal method. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. A dotted line on a phenogram indicates a negative branch length, a common product of averaging.

10

15

Fig. 16. Northern blot analysis of the expression of *gpd1*, *gpd2* and *gpd3*. A: Lane 1-3: 1.1 kb DNA fragments of *gpd1*, *gpd2* and *gpd3*, respectively. Y: RNA isolated from *M. racemosus* growing anaerobically as yeasts in the presence of glucose. M: RNA isolated from *M. racemosus* shifted from anaerobic to aerobic conditions at the time of glucose depletion and further incubated for four hours allowing the initiation of mycelial growth. Filters 1, 2 and 3 were hybridized with oligonucleotides specific for *gpd1*, *gpd2* and *gpd3*, respectively. B: Micrographs taken from the yeast (Y) and mycelial (M) culture.

20

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Fig. 17. Northern blot analysis of the expression of *gpd1*. A: Total RNA was isolated from *M. racemosus* grown aerobically in shake flask in rich medium with either glucose (Glu), glycerol (Gly) or ethanol (EtOH) added. B: Total RNA was isolated from *M. racemosus* grown anaerobically in fermentor in rich medium with either glucose or galactose. Samples were taken with 2-4 hours interval until sugar was depleted. Sugar concentrations are indicated in the boxes. After depletion of glucose, additional glucose was added and a sample was taken 1 hour after the readdition. The blots were hybridized with a 1.1 kb *gpd1* probe.

30



Fig. 18. Recombinant expression. A: Expression plasmids pEUKA4-crgA and pEUKA4-gox1 (EMBL accession nos. AJ305344 and AJ305345, respectively). B: *M. racemosus* R7B transformed with pEUKA4-crgA, and as a control R7B carrying pEUKA4-gox1, grown in the dark for 3 days. Arrows indicate yellow colonies. C: Zymogram of culture supernatants. *M. racemosus* harboring the expression plasmid pEUKA4-gox1 was inoculated in SIV medium containing 2% or 5% glucose and incubated under aerobic conditions. Samples were taken after 40 hours of incubation where glucose had not yet been depleted. GOX activity in culture supernatants was analyzed by native gel electrophoresis and staining for enzyme activity.

Fig. 19. Zymogram of culture supernatants. *M. racemosus* R7B harboring the expression plasmid pEUKA4-gox1 was inoculated in 6xSIV medium containing 5% glucose and grown in fermentors: A: under anaerobic conditions, B: initially anaerobic conditions, then shifted to aerobic conditions (the dotted line separates samples taken before and after the shift), C: under aerobic conditions. Samples were taken between 16 and 45 hours after inoculation. GOX activity in culture supernatants was analyzed by native gel electrophoresis and staining for enzyme activity. Biomass (g dry weight pr. kg culture) and glucose concentration (g/l) of the culture was determined for each sample.

Fig. 20. Expression plasmid pEUKA8-gox1

Fig. 21. Zymogram of culture supernatants. *M. racemosus* R7B harboring the expression plasmid pEUKA8-gox1 was inoculated in 6xSIV medium containing 5% glucose and grown in fermentors: A: under anaerobic conditions, B: initially anaerobic conditions, then shifted to aerobic conditions (the dotted line separates samples taken before and after the shift), C: under aerobic conditions. Samples were taken between 18 and 60 hours after inoculation. GOX activity in culture supernatants was analyzed by native gel electrophoresis and staining for enzyme activity. Commercial GOX (C) was used as positive control. Biomass (g dry weight pr. kg culture) was determined for each sample.

## Detailed Description of the Invention

The commercial use of any recombinant protein largely depends on the ability to achieve efficient production in large-scale fermentation, and productivity is limited by a number of factors in industrial fermentation of fungi.

5 The common problems associated with the use of filamentous fungi as hosts are related to the relatively high viscosity as compared to unicellular organisms, such as *Saccharomyces cerevisiae* and *Bacillus* sp., and the often very heterogeneous distribution of mycelium in dense aggregates causing a majority of the mycelium to starve, due to lack of O<sub>2</sub> and/or nutrient diffusion to all of the cells.

10

The high viscosity reduces the oxygen transfer rate that can be reached in the fermentor, which in turn adversely effects the overall energy the cells can produce, thereby leading to lower concentration of obtainable productive biomass and lower final product yield or longer fermentation times. It can therefore be seen that simply  
15 increasing biomass is not, without the proper morphology that leads to low viscosity, adequate to increase yield in fermentation. There must be an increase in productive biomass in order for any advantages to be obtained.

Dimorphic fungi do indeed exhibit certain growth characteristics, which render them  
20 suitable for culturing in fermentors. This group of fungi has the ability to reversibly switch between yeast and filamentous growth, typically as a response to a number of environmental stimuli. However, many of these organisms are relatively poorly characterized genetically and knowledge about the pathways controlling the dimorphic shift is very limited.

25

The present invention encompasses any dimorphic fungal cell, which can be used for the fermentation procedure as defined above. By "dimorphic fungal cell" is meant any fungal cell taxonomically belonging to the Eumycotina, including Zygomycetes, which is capable of displaying either a unicellular, essentially spherical morphology  
30 and/or a filamentous morphology characterised by a mycelium. This includes, but is not limited to, members of the genera Mucor. Further examples of taxonomic equivalents and other useful species can be found, for example, in Cannon, Mycopathologica 111: 75-83, 1990; Moustafa et al., Persoonia 14: 173-175, 1990; Stalpers, Stud. Mycol. 24, 1984; Upadhyay et al., Mycopathologia 87: 71-80, 1984;  
35 Subramanian et al., Cryptog. Mycol. 1: 175-185, 1980; Guarro et al., Mycotaxon 23:

419-427, 1985; Awao et al., Mycotaxon 16: 436-440, 1983; von Klopotek, Arch. Microbiol. 98:365-369, 1974; and Long et al., 1994, ATCC Names of Industrial Fungi, ATCC, Rockville, Md. Those skilled in the art will readily recognize the identity of appropriate equivalents.

5

As the results presented in the examples show, several strains may possess the morphology required to make them useful in the fermentation procedure described herein. Thus, it is understood that the utility is not limited to a single isolate or strain, but rather is a characteristic of a group of species. Those skilled in the art will recognize that other strains or isolates of these species can also be used in expression of heterologous expression. Many strains of *Mucor racemosus* species are publicly available in the collections of the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville Md. 20852.

15

Suitability of other dimorphic fungal hosts for use in fermentors can be determined by the methods described in the following examples. Briefly, candidate fungi are cultured on standard growth medium such as salts/yeast extract, soy, potato protein, or any medium supplemented with glucose or other appropriate carbon source. The fermentation is carried out at a pH of about 4-7 and at a temperature of from about 25°C to 35°C. It will be recognized that the temperature of the control fermentation should be that which is optimal for the control strain; for *M. racemosus*, this is about 28°C.

25

Useful fungal strains should be able to switch between a unicellular, essentially spherical morphology and a filamentous morphology characterised by a mycelium in response to a variety of environmental and nutritional conditions. Confirmation of utility is best determined in fermentors, by evaluating actual viscosity of the culture at various time points in the fermentation. Viscosity determination can be made by any means known in the art, e.g., Brookfield rotational viscometry (defined or unlimited shear distance and any type of spindle configuration), kinematic viscosity tubes (flow-through tubes), falling ball viscometer or cup-type viscometer.

30

As noted above, *Mucor racemosus* is, because of its excellent dimorphic morphology, among the preferred species for use in recombinant protein production.

35

However, this species, in anaerobic cultures grown without nutrient limitation adopts

a unicellular, essentially spherical morphology that results in low biomass production. This is due, among other things, to growth inhibition caused by accumulation of ethanol.

5      Aerobic growth of *Mucor racemosus* results in mycelia, which are the preferred source of production of the recombinant proteins. The mycelia of filamentous fungi have a naturally high capacity for protein secretion due to their saprophytic lifestyle, and protein secretion in filamentous fungi occurs at the hyphal tips (Gordon et al 2000, *Microbiology* **146**: 415-426; Wösten et al 1991, *J. Gen. Microbiology*  
10      **137**:2017-2023; Schauwecker et al 1995).

The present invention combines the advantages of both of the above-mentioned growth morphologies without compromising biomass production or protein secretion.

15      Fungal cells the morphology of which is regulatable by the regulator of morphology

When the present invention in one aspect relates to an isolated polynucleotide comprising

- 20      i)      a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- 25      ii)      a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

wherein the first and second nucleotide sequences are not natively associated,

30      the dimorphic fungal cell, the morphology of which is regulatable by the regulator of morphology, is preferably capable of growing as i) a multinucleated cell having a unicellular, essentially spherical morphology and/or ii) a mycelium having a filamentous structure and comprising multinucleated cells. The different morphologies can be observed individually under appropriate growth conditions, and  
35      both forms of morphology can be observed in connection with a dimorphic shift. The

unicellular dimorphic fungal cells are in one embodiment multinucleated and optionally multipolar, but they may also be bipolar or monopolar.

5 It will be understood that the dimorphic fungal cells mentioned herein immediately above and below are in one embodiment fungal cells, the morphology of which is regulatable by the regulator of morphology. Such fungal cells thus characterise in functional terms the polynucleotide according to the invention comprising the first nucleotide sequence encoding the regulator of morphology and the second nucleotide sequence comprising the expression signal, wherein said first and  
10 second nucleotide sequences are not natively associated.

In another embodiment, the dimorphic cells listed herein below are also comprised by the term "host cell", wherein such a host cell is transfected or transformed with the polynucleotide according to the invention comprising said first and second not  
15 natively associated nucleotide sequences, wherein said second nucleotide sequence is capable of directing expression of the first nucleotide sequence in such a host cell. Furthermore, "host cells" shall also be understood to comprise fungal cells that are not dimorphic fungal cells.

20 When regulating the morphology of a dimorphic fungal cell, the increased or decreased production of regulator of morphology preferably results in a dimorphic shift of the dimorphic fungal cell and/or in a filamentous morphology of the dimorphic fungal cell and/or an improved filamentation of the dimorphic cell.

25 When regulating the morphology of a fungal cell that is not a dimorphic cell, the increased or decreased production of regulator of morphology in such a fungal cell preferably results in a filamentous morphology of the fungal cell and/or an improved filamentation of the fungal cell.

30 Improved or increased filamentation of a fungal cell including a dimorphic fungal cell is defined herein as any one of i) the transition of a single cell from a unicellular, essentially spherical morphology to a mycelium having a filamentous morphology; ii) an increase in the proportion of cells having a filamentous morphology; iii) an increased branching frequency (hyperbranching); and iv) a decreased hyphal growth  
35 unit (HGU) length.

The HGU is the total length (or area) of a hyphal element divided by the number of tips. The growth unit of a mycelium clearly differs qualitatively from the “growth units” (i.e. cells) of a unicellular fungal cell having an essentially spherical morphology. Branch initiation in the mycelium of a filamentous fungal cell is regulated during filamentous growth. When the mean HGU value of a mycelium (total hyphal length of the mycelium divided by its total number of tips) exceeds a critical value, a new branch is initiated. A unicellular fungal cell having an essentially spherical morphology is defined as having an HGU of 0, and such a cell will only obtain a HGU if it starts to produce filaments. Increased branching will result in a decrease of HGU.

There is a linear relation between the HGU length and culture viscosity (Bocking et al. 1999, *Biotechnol. Bioeng.* **65**: 638-48). According to one hypothesis, the number of hyphal tips per unit biomass is maximal at early stages of filamentous growth, whereas at later times their numbers do not increase in proportion to the biomass (postulated but not experimentally shown in Chaudhuri et al 1999, *FEMS Microbiol. Lett.* **177**: 39-45; Trinci 1974).

Consequently, the regulator of morphology can be expressed in both a dimorphic fungal cell and a fungal cell that is not capable of displaying dimorphic morphology. The result of regulating the production of the regulator is preferably an improved filamentation, and such an improved filamentation in one embodiment occurs in a dimorphic fungal cell independently of a dimorphic shift. This will e.g. be the case when the regulator is being produced in a dimorphic cell growing as a mycelium or adopting a filamentous morphology, wherein said increased or decreased production of regulator of morphology results in an improved filamentation. An increased expression or a decreased expression is an expression that is altered as compared to the expression of the regulator directed by the native expression signal of said regulator.

In one embodiment, the dimorphic fungal cell, the morphology of which is regulatable by the regulator of morphology, belongs to the class of Zygomycetes, including the order of Mucorales, including from the order of Mucorales a genus selected from the group of genera consisting of *Mucor*, *Thermomucor*, *Rhizomucor*,

Mycotypha, Rhizopus, and Cokeromyces, including Cokeromyces recurvatus. Another preferred dimorphic fungal cell is Yarrowia lipolytica.

- It is preferred in one embodiment that the dimorphic fungal cell, the morphology of which is regulatable by the regulator of morphology, belongs to the genus *Mucor*. Preferred *Mucor* species includes, but is not limited to *M. racemosus*, *M. hiemalis*, *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and *M. subullissimus*. *M. racemosus* is particularly preferred.
- Additionally preferred *Mucor* species, the morphology of which is regulatable by the regulator of morphology, includes, but is not limited to *Mucor abundans*, *Mucor adventitus* = Syn. of *Mucor hiemalis* f. *hiemalis*, *Mucor adventitus* var. *aurantiacus* = Syn. of *Mucor hiemalis* f. *hiemalis*, *Mucor alboater* = Syn. of *Mucor piriformis*, *Mucor aligarensis*, *Mucor amphibiorum*, *Mucor angulisporus* = Syn. of *Mortierella ramanniana* var. *angulispora*, *Mucor ardhlaengiktus*, *Mucor aromaticus* = Syn. of *Mucor recurvus* var. *recurvus*, *Mucor assamensis* = Syn. of *Hyphomucor assamensis*, *Mucor attenuatus* = Syn. of *Mucor flavus*, *Mucor azygosporus*, *Mucor bacilliformis*, *Mucor bainieri*, *Mucor bedrchanii* = Syn. of *Mucor fuscus*, *Mucor botryoides* = Syn. of *Actinomucor elegans*, *Mucor botryoides* var. *minor* = Syn. of *Actinomucor elegans*, *Mucor brunneogriseus* = Syn. of *Mucor plumbeus*, *Mucor brunneus* = Syn. of *Mucor plumbeus*, *Mucor buntingii* = Syn. of *Rhizomucor pusillus*, *Mucor chibinensis* = Syn. of *Mucor racemosus* f. *chibinensis*, *Mucor christianiensis* = Syn. of *Mucor racemosus* f. *racemosus*, *Mucor circinans* = Syn. of *Pirella circinans*, *Mucor circinelloides* f. *circinelloides*, *Mucor circinelloides* f. *griseocyanus*, *Mucor circinelloides* f. *janssenii*, *Mucor circinelloides* f. *lusitanicus*, *Mucor coprophilus* = Syn. of *Mucor mucedo*, *Mucor corticolus* = Syn. of *Mucor hiemalis* f. *corticolus*, *Mucor corymbifer* = Syn. of *Absidia corymbifera*, *Mucor cunninghamelloides* = Syn. of *Actinomucor elegans*, *Mucor cylindrosporus* = Syn. of *Mucor microsporus*, *Mucor dimorphosporus* = Syn. of *Mucor racemosus* f. *racemosus*, *Mucor dispersus* = Syn. of *Backusella lamprospora*, *Mucor dispersus* var. *megalosporus* = Syn. of *Mucor zychae* var. *linnemanniae*, *Mucor dubius* = Syn. of *Mucor circinelloides* f. *circinelloides*, *Mucor falcatus*, *Mucor flavus*, *Mucor fragilis*, *Mucor fuscus*, *Mucor genevensis*, *Mucor gigasporus*, *Mucor globosus* = Syn. of *Mucor racemosus* f. *sphaerosporus*, *Mucor glomerula* = Syn. of *Actinomucor elegans*, *Mucor grandis*, *Mucor griseobrunneus* = Syn. of *Mucor fuscus*, *Mucor griseocyanus* = Syn. of *Mucor*

- circinelloides f. griseocyanus, *Mucor griseocyanus* f. *janssenii* = Syn. of *Mucor*  
*circinelloides* f. *janssenii*, *Mucor griseolilacinus* = Syn. of *Mucor circinelloides* f.  
*lusitanicus*, *Mucor griseoochraceus* = Syn. of *Mucor mucedo*, *Mucor*  
*griseoochraceus* var. *minuta* = Syn. of *Mucor minutus*, *Mucor griseoroseus* = Syn. of  
 5 *Mucor circinelloides* f. *circinelloides*, *Mucor guilliermondii*, *Mucor hiemalis*, *Mucor*  
*hiemalis* f. *corticulus*, *Mucor hiemalis* f. *hiemalis*, *Mucor hiemalis* f. *luteus*, *Mucor*  
*hiemalis* f. *silvaticus*, *Mucor hiemalis* var. *albus* = Syn. of *Mucor hiemalis* f. *hiemalis*,  
*Mucor hiemalis* var. *flavus* = Syn. of *Mucor hiemalis* f. *hiemalis*, *Mucor hiemalis* var.  
*griseus* = Syn. of *Mucor hiemalis* f. *hiemalis*, *Mucor hiemalis* var. *toundrae* = Syn. of  
 10 *Mucor hiemalis* f. *hiemalis*, *Mucor humicolus* = Syn. of *Mucor hiemalis* f. *hiemalis*,  
*Mucor inaequisporus*, *Mucor indicae-seudaticae* = Syn. of *Thermomucor indicae-*  
*seudaticae*, *Mucor indicus*, *Mucor janssenii* = Syn. of *Mucor circinelloides* f.  
*janssenii*, *Mucor jauchae* = Syn. of *Mucor circinelloides* f. *lusitanicus*, *Mucor*  
*javanicus* = Syn. of *Mucor circinelloides* f. *circinelloides*, *Mucor kanivcevi* = Syn. of  
 15 *Mucor strictus*, *Mucor kurssanovii* = Syn. of *Mucor circinelloides* f. *janssenii*, *Mucor*  
*lamprosporus* = Syn. of *Backusella lamprospora*, *Mucor lausannensis* = Syn. of  
*Mucor hiemalis* f. *hiemalis*, *Mucor laxorrhizus*, *Mucor laxorrhizus* var. *ovalisporus*,  
*Mucor lusitanicus* = Syn. of *Mucor circinelloides* f. *lusitanicus*, *Mucor luteus* = Syn. of  
*Mucor hiemalis* f. *luteus*, *Mucor luteus* var. *indica* = Syn. of *Mucor variisporus*,  
 20 *Mucor mandshuricus* = Syn. of *Mucor circinelloides* f. *circinelloides*, *Mucor mephitis*  
 = Syn. of *Mucor flavus*, *Mucor meridionalis* = Syn. of *Mucor flavus*, *Mucor*  
*microsporus*, *Mucor miehei* = Syn. of *Rhizomucor miehei*, *Mucor minutus*, *Mucor*  
*mirus* = Syn. of *Mortierella isabellina*, *Mucor mousanensis*, *Mucor mucedo*, *Mucor*  
*mucilagineus* = Syn. of *Mucor plasmaticus*, *Mucor murorum* = Syn. of *Mucor*  
 25 *mucedo*, *Mucor nanus*, *Mucor norvegicus* = Syn. of *Rhizopus oryzae*, *Mucor*  
*oblongiellipticus*, *Mucor oblongisporus*, *Mucor odoratus*, *Mucor ovalisporus* = Syn. of  
*Mucor aligarensis*, *Mucor peacockensis* = Syn. of *Mucor flavus*, *Mucor petrinsularis*  
 = Syn. of *Mucor fuscus*, *Mucor petrinsularis* var. *echinosporus* = Syn. of *Mucor*  
*fuscus*, *Mucor petrinsularis* var. *ovalisporus* = Syn. of *Mucor aligarensis*, *Mucor*  
 30 *pirelloides* = Syn. of *Pirella circinans*, *Mucor piriformis*, *Mucor pispekii* = Syn. of  
*Mucor racemosus* f. *racemosus*, *Mucor plasmaticus*, *Mucor plumbeus*, *Mucor*  
*plumbeus* var. *globosus* = Syn. of *Mucor racemosus* f. *sphaerosporus*, *Mucor*  
*plumbeus* var. *intermedius* = Syn. of *Mucor fuscus*, *Mucor plumbeus* var. *levisporus*  
 = Syn. of *Mucor racemosus* f. *sphaerosporus*, *Mucor prainii* = Syn. of *Mucor*  
 35 *circinelloides* f. *circinelloides*, *Mucor prayagensis*, *Mucor pseudolamprosporus* =



Syn. of *Backusella circina*, *Mucor psychrophilus*, *Mucor pusillus* = Syn. of *Rhizomucor pusillus*, *Mucor pyri* = Syn. of *Mucor racemosus* f. *sphaerosporus*, *Mucor racemosus* f. *brunneus* = Syn. of *Mucor racemosus* f. *racemosus*, *Mucor racemosus* f. *chibinensis*, *Mucor racemosus* f. *racemosus*, *Mucor racemosus* f. *sphaerosporus*, *Mucor ramannianus* = Syn. of *Mortierella ramanniana* var. *ramanniana*, *Mucor ramificus* = Syn. of *Mucor circinelloides* f. *circinelloides*, *Mucor ramiger* = Syn. of *Mucor fuscus*, *Mucor ramosissimus*, *Mucor recurvus*, *Mucor recurvus* var. *indicus*, *Mucor recurvus* var. *recurvus*, *Mucor rouxianus* = Syn. of *Mucor indicus*, *Mucor rouxii*, *Mucor rufescens* = Syn. of *Mucor odoratus*, *Mucor saturninus*, *Mucor saximontensis* = Syn. of *Zygorhynchus moelleri*, *Mucor sciurinus* = Syn. of *Mucor flavus*, *Mucor silvaticus* = Syn. of *Mucor hiemalis* f. *silvaticus*, *Mucor sinensis*, *Mucor sphaerosporus* = Syn. of *Mucor racemosus* f. *sphaerosporus*, *Mucor spinescens* = Syn. of *Mucor plumbeus*, *Mucor spinosus* = Syn. of *Mucor plumbeus*, *Mucor strictus*, *Mucor subtilissimus*, *Mucor tauricus* = Syn. of *Rhizomucor tauricus*, *Mucor tenellus* = Syn. of *Mucor circinelloides* f. *janssenii*, *Mucor thermophilus*, *Mucor tuberculisporus*, *Mucor ucrainicus*, *Mucor vallesiacus* = Syn. of *Mucor hiemalis* f. *hiemalis*, *Mucor variabilis*, *Mucor varians* = Syn. of *Mucor racemosus* f. *racemosus*, *Mucor variisporus*, *Mucor vesiculosus* = Syn. of *Gongronella butleri*, *Mucor wosnessenskii* = Syn. of *Mucor piriformis*, *Mucor zeicola* = Syn. of *Mucor circinelloides* f. *lusitanicus*, *Mucor zonatus*, *Mucor zychae* var. *linnemanniae*, and *Mucor zychae* var. *zychae* ("=" denotes "same as").

Although dimorphic fungal cells represent one group of fungal cells, the morphology of which is regulatable by the regulator of morphology, the present invention is not limited to dimorphic fungal cells as host cells for the regulator of morphology. The invention also relates to host cells in the form of any other fungal cell including any filamentous form of the subdivision Eumycotina. The fungal cells are characterized by a vegetative mycelium composed of chitin, cellulose, and other complex polysaccharides. Vegetative growth by filamentous fungi is by hyphal elongation. In contrast, vegetative growth by yeasts such as *S. cerevisiae* is by budding of a unicellular thallus. The filamentous fungi of the present invention are thus morphologically, physiologically, and genetically distinct from yeasts. Also, recent illustrations of differences between *S. cerevisiae* and filamentous fungi include the inability of *S. cerevisiae* to process *Aspergillus* and *Trichoderma* introns and the inability to recognize many transcriptional regulators of filamentous fungi.

- Various species of filamentous fungi may be used as host cells in accordance with the present invention, including the following genera: *Aspergillus*, including *Aspergillus niger* and *Aspergillus oryzae*, *Trichoderma*, *Neurospora*, *Podospora*, *Endothia* *Mucor*, *Cochiobolus* and *Pyricularia*. Specific expression hosts include *A. nidulans* (Yelton, M., et al., 1984, *Proc. Natl. Acad. Sci. USA*, 81:1470-1474; Mullaney, E. J. et al., 1985, *Mol. Gen. Genet.*, 199:37-45; John, M. A. and J. F. Peberdy, 1984, *Enzyme Microb. Technol.*, 6:386-389; Tilburn, et al., 1982, *Gene*, 26:205-221; Ballance, D. J., et al., 1983, *Biochem. Biophys. Res. Comm.*, 112:284-289; and Johnston, I. L., et al., 1985, *EMBO J.*, 4:1307-1311), *A. niger* (Kelly, J. M. and M. Hynes, 1985, *EMBO*, 4:475-479), *A. awomari*, e.g., NRRL 3112, ATCC 22342 (NRRL 3112), ATCC 44733, ATCC 14331 and strain UVK 143f, *A. oryzae*, e.g., ATCC 11490, *N. crassa* (Case, M. E., et al., 1979, *Proc. Natl. Acad. Sci. USA*, 76:5259-5263; and Lambowitz U.S. Pat. No. 4,486,533; Kinsey, J. A. and J. A. Rambosek, 1984, *Molecular and Cellular Biology* 4:117-122; Bull, J. H. and J. C. Wooton, 1984, *Nature*, 310:701-704); *Trichoderma reesei*, e.g. NRRL 15709, ATCC 13631, 56764, 56765, 56466, 56767, and *Trichoderma viride*, e.g., ATCC 32098 and 32086.
- Further preferred fungal host cells are cells of the genus *Cunninghamella*, including *Cunninghamella elegans* and *Cunninghamella polymorpha*, cells of the genus *Rhizomucor*, including *Rhizomucor miehei*, *Rhizomucor pusillus*, *Rhizomucor variabilis*, *Rhizomucor variabilis*, and *Rhizomucor variabilis*, cells of the genus *Rhizopus*, including *Rhizopus oryzae*, *Rhizopus microsporus* var. *oligosporus*, and *Rhizopus niveus*, cells of the genus *Mortierella*, including *Mortierella isabelina*, *Mortierella verticillata*, *Mortierella alpina*, and *Mortierella vinacea*.

In another embodiment, there is provided a polynucleotide comprising

- i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto

- ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

5 wherein the first and second nucleotide sequences are not natively associated, and wherein the dimorphic fungal cell, the morphology of which is regulatable by the regulator of morphology, is capable of growing as a uninucleated cell having a unicellular, essentially spherical morphology, and/or capable of growing as a filamentous structure comprising uninucleated cells. Examples of such dimorphic  
10 fungi includes, but is not limited to Yarrowia, Candida and Arxula.

#### Origin of the first and/or second nucleotide sequence

15 In yet another preferred embodiment, there is provided a polynucleotide comprising

- i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- 20 ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

25 wherein the first and second nucleotide sequences are not natively associated, and

wherein said first and/or second nucleotide sequence is derived from a microbial cell, including a microbial cell selected from the group of microbial cells consisting of eukaryotic microbial cells and procaryotic microbial cells.

30 When the microbial cell from which the first and/or second nucleotide sequence is derived is a eukaryotic microbial cell, it is preferably selected from the group of eukaryotic cells consisting of fungal cells and yeast cells.

35 In one preferred embodiment, the eukaryotic microbial cell from which the first and/or second nucleotide sequence is derived is a fungal cell, including a

filamentous fungal cell, including a dimorphic fungal cell, including dimorphic fungal cells capable of growing as a multinucleated cell having a unicellular, essentially spherical morphology and/or capable of growing as a mycelium having a filamentous structure and comprising multinucleated cells, including a fungal cell  
 5 belonging to the class of Zygomycetes, including a fungal cell belonging to the order of Mucorales, including a fungal cell belonging to the genus selected from the group of genera consisting of Mucor, Thermomucor, Rhizomucor, Mycotypha, Rhizopus and Cokeromyces, including Cokeromyces recurvatus, including a fungal cell belonging to the genus Mucor, including a fungal cell selected from the group of  
 10 Mucor species consisting of *M. racemosus*; *M. hiemalis*, *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and *M. subullissimus*, including a fungal cell such as *M. racemosus*.

In another preferred embodiment, the eukaryotic microbial cell from which the first and/or second nucleotide sequence is derived is a dimorphic fungal cells capable of  
 15 growing as a uninucleated cell having a unicellular, essentially spherical morphology, and/or capable of growing as a filamentous structure comprising uninucleated cells. Examples of such dimorphic fungi includes, but is not limited to *Yarrowia*, *Candida* and *Arxula*.

20 Vector comprising the polynucleotide encoding the regulator of morphology

The polynucleotide according to the invention comprising

- 25 i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic  
 30 fungal cell,

wherein the first and second nucleotide sequences are not natively associated, is in one embodiment located on a extrachromosomal, recombinant DNA molecule, preferably in the form of an expression vector, which may further comprising a signal  
 35 sequence encoding a signal peptide, wherein the signal sequence is operably linked

to the coding sequence of the first nucleotide sequence. The recombinant DNA molecule preferably further comprises a selectable marker, and optionally a genetic element, preferably a transposon, capable of mediating transposition of the recombinant DNA molecule. In another embodiment, the first and second nucleotide sequences are chromosomally located. Artificial chromosomes, including BACs and YACs, are included in the term vector as used herein.

### Regulators of morphology

A regulator of morphology as used herein includes any polypeptide the recombinant production of which in a fungal cell, including a dimorphic fungal cell, results in an altered filamentation including an increased or decreased filamentation, as compared to the filamentation observed when the regulator is encoded by a polynucleotide operably linked to its native expression signal.

It is preferred that the recombinant production in a fungal cell, including a dimorphic fungal cell, of the regulator of morphology results in an increased filamentation. In the case of a dimorphic fungal cell, the production of the regulator of morphology generates in one preferred embodiment an increased filamentation by inducing a dimorphic shift from i) a predominant yeast-like morphology characterised in one embodiment by a multinucleated cell having a unicellular, essentially spherical morphology to ii) a predominant filamentous structure characterised by a mycelium comprising multinucleated cells.

Example 1 is a study of the morphologies associated with a dimorphic shift of *Mucor racemosus*. The dimorphic fungal cell *Mucor racemosus* can respond to environmental cues by growing e.g. as a multinucleated cell having a unicellular, essentially spherical morphology, or as a filamentous fungal cell characterised by a mycelium comprising multinucleated cells. Both filamentous and non-filamentous growth is organized in a single multinucleated cell. Unicellular *Mucor racemosus* cells of essentially spherical morphology are multipolar meaning that daughter cells can originate at different positions of the mother cell as opposed to bipolar and monopolar cells. The *Mucor racemosus* cells each harbor more than one nucleus, while *Mucor racemosus* mycelium is aseptate, but with evenly distributed nuclei.

Following a shift from anaerobic to aerobic conditions, a transition from non-filamentous cell morphology to filamentous structures occurs. Fig. 1 to 4 illustrate the monitoring of this morphogenetic process on single *Mucor racemosus* cells.

5 Preferred regulators in accordance with the present invention are kinases and transcription factors participating in the genetic networks regulating fungal cellular processes such as e.g. filamentation. A number of such regulators are illustrated in Fig. 1. Further preferred regulators of morphology according to the present invention are described herein below.

10

### PKAR

Example 2 discloses the cloning of the *pkar* gene encoding the regulatory subunit of the protein kinase A (PKAR) of *Mucor racemosus*. During the dimorphic shift, *i.e.*,  
 15 during the transition from yeast to filamentous growth, the expression of *pkar* increases a twofold. Also, addition of exogenous dibutyryl-cAMP (dbcAMP) during aerobic growth resulted in further induction of *pkar* expression, up to a sevenfold. These observations correlate with the 'repressor model' for the control of morphology in dimorphic fungi, where a repressor (*e.g.*, PKAR) is responsible for  
 20 aerobic filamentous growth. In this model, derepression mediated by cAMP binding to PKAR and release of the catalytic subunit of the protein kinase A leads to yeast growth.

Induction of *pkar* expression is dependent of the shift from anaerobic to aerobic  
 25 growth and independent of glucose concentration. Overexpression of *pkar* resulted in a multi-branched colony phenotype on solid medium.

Mitogen-activated protein (MAP) kinases and the transcription factor STE12 are elements of parallel signal transduction pathways involved in filamentation in  
 30 different fungi (Fig. 5). Via degenerate PCR, the present invention has identified both a gene encoding a STE12 homologue (*ste12*) and a gene encoding a MAP kinase homologue (*mpk1*; mitogen activated protein kinase 1). Further, identification of an upstream regulator, the MAP kinase kinase kinase STE20 homologue (*ste20*) is described in Example 2.

35

Accordingly, when the present invention in one aspect relates to an isolated polynucleotide comprising

- 5           i)       a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- ii)       a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic
- 10           fungal cell,

wherein the first and second nucleotide sequences are not natively associated,

the first nucleotide sequence is preferably selected from the group consisting of

- 15           i)       a polynucleotide comprising nucleotides 542 to 1930 of SEQ ID NO:1, and
- ii)       a polynucleotide comprising or essentially consisting of the coding sequence of *pkar* encoding the regulatory subunit of protein kinase A (PKAR) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14062; and
- 20           iii)       a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and
- 25           iv)       a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i) or (ii), wherein said fragment
- 30           a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the inhibitor peptide at the N-terminal end of
- 35           the regulatory subunit of *Mucor racemosus* protein kinase A, or a

cAMP binding domain of the regulatory subunit of *Mucor racemosus* protein kinase A, wherein said inhibitor peptide or said cAMP binding domain is preferably comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

5

- c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the *Mucor racemosus* catalytic subunit for protein kinase A; and

10

- v) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) (iii), and (iv), and encodes a polypeptide that

15

- a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or

20

- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the inhibitor peptide at the N-terminal end of the regulatory subunit of *Mucor racemosus* protein kinase A, or a cAMP binding domain of the regulatory subunit of *Mucor racemosus* protein kinase A, wherein said inhibitor peptide or said cAMP binding domain is preferably comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

25

- c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the *Mucor racemosus* catalytic subunit for protein kinase A; and

30

- vi) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iv) and (v),

35

and the complementary strand of such a polynucleotide.



Stringent conditions as used herein shall denote stringency as normally applied in connection with Southern blotting and hybridization as described e.g. by Southern E. M., 1975, J. Mol. Biol. 98:503-517. For such purposes it is routine practise to include

5 steps of prehybridization and hybridization. Such steps are normally performed using solutions containing 6x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA (incubation for 18 hrs at 42°C), followed by washings with 2x SSC and 0.5% SDS (at room temperature and at 37°C), and a washing with 0.1x SSC and 0.5% SDS (incubation at 68°C for 30 min), as described

10 by Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), which is incorporated herein by reference.

Accordingly, the first nucleotide sequence in one preferred embodiment preferably comprises nucleotides 542 to 1930 of SEQ ID NO:1.

15

In another embodiment, the first nucleotide sequence comprises or essentially consists of the coding sequence of *pkar* encoding the regulatory subunit of protein kinase A (PKAR) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14062.

20

In yet another embodiment, the first nucleotide sequence encodes a polypeptide having the amino acid sequence as shown in SEQ ID NO:2.

In a further embodiment, the first nucleotide sequence encodes a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:2, wherein said fragment

25

- a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
  - b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the inhibitor peptide at the N-terminal end of the regulatory subunit of *Mucor racemosus* protein kinase A, or a cAMP binding domain of the regulatory subunit of *Mucor racemosus* protein kinase A, wherein said inhibitor peptide or said cAMP binding
- 30
- 35

domain is preferably comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or

- 5 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the *Mucor racemosus* catalytic subunit for protein kinase A; and

10 In a still further embodiment, the first polynucleotide sequence comprises a polynucleotide the complementary strand of which hybridizes under stringent conditions with a polypeptide that

- 15 a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the inhibitor peptide at the N-terminal end of the regulatory subunit of *Mucor racemosus* protein kinase A, or a cAMP binding domain of the regulatory subunit of *Mucor racemosus* protein kinase A, wherein said inhibitor peptide or said cAMP binding domain is preferably comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
- 20 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the *Mucor racemosus* catalytic subunit for protein kinase A.
- 25

30 The regulatory subunit of protein kinase A consists of an inhibitor peptide at the N-terminal end (around position 91-112) that occupies the active site of the catalytic subunit (PKAC) in the holoenzyme form of the protein, keeping the PKAC subunit from phosphorylating any substrate molecules. Two cAMP binding domains called A and B are also present in PKAR. When cAMP binds to these sites, the molecule undergoes a conformational change, withdrawing the inhibitor peptide from PKAC

and allowing PKAR and PKAC to separate. At low cAMP levels, PKAR reverts to its original conformation and binds to PKAC.

5 Polyclonal antibodies against PKAR exist, and some bind to PKAR irrespective of its binding to PKAC. Standard cAMP measurements are also well known to the skilled person.

10 There is also provided a polynucleotide comprising a first nucleotide sequence which is degenerate to any of the sequences described herein immediately above, as well as a polynucleotide comprising a first nucleotide sequence in the form of the the complementary strand of polynucleotides described herein immediately above.

#### STE20

15 STE20 is an upstream regulator in the MAP kinase transduction pathway. When the present invention in one aspect relates to an isolated polynucleotide comprising

- 20 i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

25 wherein the first and second nucleotide sequences are not natively associated,

the first nucleotide sequence is preferably selected from the group consisting of

- 30 i) a polynucleotide comprising nucleotides 1 to 634 of SEQ ID NO:3, and
- ii) a polynucleotide comprising or essentially consisting of the coding sequence of *ste20* encoding a MAP kinase kinase kinase (STE20) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14065; and

- iii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and
- 5 iv) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i) or (ii), wherein said fragment
  - a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
  - 10 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or
  - 15 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and
  - 20
- v) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) (iii), and (iv), and encodes a polypeptide that
- 25
  - a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
  - b) is recognised by an antibody, or a binding fragment thereof, which is
  - 30 capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or

c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and

5

vi) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iv) and (v),

10

and the complementary strand of such a polynucleotide.

Accordingly, the first nucleotide sequence in one preferred embodiment preferably comprises nucleotides 1 to 634 of SEQ ID NO:3.

15

In another embodiment, the first nucleotide sequence comprises or essentially consists of the coding sequence of *ste20* encoding a MAP kinase kinase kinase (STE20) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14065.

20

In yet another embodiment, the first nucleotide sequence encodes a polypeptide having the amino acid sequence as shown in SEQ ID NO:4.

In a further embodiment, the first nucleotide sequence encodes a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:4, wherein

25

said fragment

a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

30

b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or

35

- c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.

5

In a still further embodiment, the first polynucleotide sequence comprises a polynucleotide the complementary strand of which hybridizes under stringent conditions with a polypeptide that

- 10 a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or
- 15 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.
- 20

25 There is also provided a polynucleotide comprising a first nucleotide sequence which is degenerate to any of the sequences described herein immediately above, as well as a polynucleotide comprising a first nucleotide sequence in the form of the the complementary strand of polynucleotides described herein immediately above.

30 The term catalytic domain as used herein above shall include the STE20 'conserved' structural motif (the catalytic domain of Serine/Threonine kinases) as shown below:

Length = 296  
Score = 85.1 bits (209), Expect = 2e-18

35 Mucor: ETALMALSKHPHVLRVYGSFVH-----GSKLYIVTPYMAVGSCLDIMKLSFPDGLDEIS  
Sbjct: 48 ELVLMKCVNHKNIIISLLNVFTPQKTLEEFQDVYLVME LM-DANLCQVIQMELD-----HER 102

Mucor: IATILKQALEGLAYLHKNGHIHRDVKAGNLLMDEDGSVLLAD  
Sbjct:103 MSYLLYQMLCGIKHLHSAGIIHRDLKPSNIVVKSDCTLKILD 144

Besides being capable of recognising the catalytic domain of *Mucor racemosus* STE20, or a fragment thereof, the antibody, or a binding fragment thereof, may also  
 5 recognise other motifs present in the primary amino acid sequence of STE20.  
 Examples of such motifs are listed herein below:

Many putative downstream effectors of the small GTPases Cdc42 and Rac contain  
 a GTPase binding domain (GBD), also called p21 binding domain (PBD), which has  
 10 been shown to specifically bind the GTP bound form of Cdc42 or Rac, with a  
 preference for Cdc42. The most conserved region of GBD/PBD domains is the N-  
 terminal Cdc42/Rac interactive binding motif (CRIB), which consists of about 16  
 amino acids with the consensus sequence I-S-X-P-X(2,4)-F-X-H-X(2)-H-V-G.  
 Although the CRIB motif is necessary for the binding to Cdc42 and Rac, it is not  
 15 sufficient to give high-affinity binding.

A less well conserved inhibitory switch (IS) domain responsible for maintaining the  
 proteins in a basal (autoinhibited) state is located C-terminally of the CRIB- motif.  
 GBD domains can adopt related but distinct folds depending on context. Although  
 20 GBD domains are largely unstructured in the free state, the IS domain forms an N-  
 terminal beta hairpin that immediately follows the conserved CRIB motif and a  
 central bundle of three alpha helices in the autoinhibited state. The interaction  
 between GBD domains and their respective G proteins leads to the formation of a  
 high-affinity complex in which unstructured regions of both the effector and the G  
 25 protein become rigid. CRIB motifs from various GBD domains interact with Cdc42 in  
 a similar manner, forming an intermolecular beta-sheet with strand beta-2 of Cdc42.  
 Outside the CRIB motif, the C-termini of the various GBD domains are very diver-  
 gent and show variation in their mode of binding to Cdc42, perhaps determining the  
 specificity of the interaction. Binding of Cdc42 or Rac to the GBD domain causes a  
 30 dramatic conformational change, refolding part of the IS domain and unfolding the  
 rest.

Proteins known to contain a GBD domain include mammalian activated Cdc42-  
 associated kinases (ACKs), nonreceptor tyrosine kinases implicated in integrin-  
 35 coupled pathways, mammalian p21-activated kinases (PAK1 to PAK4), ser-  
 ine/threonine kinases that modulate cytoskeletal assembly and activate MAP-kinase

pathways and yeast STE20, homologue of mammalian PAKs. STE20 is involved in the mating/pheromone MAP kinase cascade.

### MPK1

5

Mitogen-activated protein kinases (MAP kinases) form a group of serine/threonine protein kinases that play important roles in signal transduction pathways regulating adaptative response to a wide range of stimuli.

10

When the present invention in one aspect relates to an isolated polynucleotide comprising

15

- i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

20

wherein the first and second nucleotide sequences are not natively associated,

the first nucleotide sequence is preferably selected from the group consisting of

25

- i) a polynucleotide comprising nucleotides 1 to 541 of SEQ ID NO:5, and
- ii) a polynucleotide comprising or essentially consisting of the coding sequence of *mpk1* encoding mitogen activated protein kinase 1 of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14063 and
- iii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and
- iv) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i) or (ii), wherein said fragment

35



- a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 5 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or
- 10 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and
- 15 v) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii), (iii), and (iv), said polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:6, or a fragment thereof, wherein said
- 20 fragment
- a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 25 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or
- 30 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and
- 35

a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iv) and (v),

and the complementary strand of such a polynucleotide.

5

Accordingly, the first nucleotide sequence in one preferred embodiment preferably comprises nucleotides 1 to 541 of SEQ ID NO:5.

10

In another embodiment, the first nucleotide sequence comprises or essentially consists of the coding sequence of *mpk1* encoding mitogen activated protein kinase 1 of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14063.

15

In yet another embodiment, the first nucleotide sequence encodes a polypeptide having the amino acid sequence as shown in SEQ ID NO:6.

20

In a further embodiment, the first nucleotide sequence encodes a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:6, wherein said fragment

25

a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or

30

c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and

In a still further embodiment, the first polynucleotide sequence comprises a polynucleotide the complementary strand of which hybridizes under stringent conditions with a polypeptide that

- 5                   a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen  
10                   activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or
- c) is competing with a polypeptide comprising or essentially consisting of  
15                   the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and

20                   The term catalytic domain as used herein above shall include the conserved TXY motif in which both the threonine and tyrosine residues are phosphorylated during activation of the enzyme by upstream dual-specificity MAP kinase kinases (MAPKKs). In addition to the TXY motif, other motifs include the region located just after the TXY motif and containing a F and a C residue that are MAPK-specific. The R and E residues in the first part of the pattern, and the R, D and K residues in the  
25                   second part, are shared by many additional protein kinases. They have been included in the pattern to eliminate matches from unrelated sequences in the database, and to "anchor" the MAPK-specific F and C residues to this region. Accordingly, one preferred catalytic domain comprises the consensus pattern: F-x(10)-R-E-x(72,86)-R-D-x-K-x(9)-C, and this domain is preferably recognised by an  
30                   antibody used to define fragments of MAPK in accordance with the present invention.

There is also provided a polynucleotide comprising a first nucleotide sequence which is degenerate to any of the sequences described herein immediately above,

as well as a polynucleotide comprising a first nucleotide sequence in the form of the complementary strand of polynucleotides described herein immediately above.

### STE12

5

When the present invention in one aspect relates to an isolated polynucleotide comprising

10

i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto

15

ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

wherein the first and second nucleotide sequences are not natively associated,

20

the first nucleotide sequence is preferably selected from the group consisting of

25

i) a polynucleotide comprising nucleotides 1 to 384 of SEQ ID NO:7, and

ii) a polynucleotide comprising or essentially consisting of the coding sequence of *ste12* encoding a transcription factor of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14064; and

30

iii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:8; and

iv) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i) or (ii), wherein said fragment

35

a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

- 5                   b) is recognised by an antibody, or a binding fragment thereof, which is  
i) capable of recognising an N-terminal region of STE12 involved in  
polynucleotide binding and/or ii) capable of recognising an induction  
domain located in the central region of STE12 and/or iii) capable of  
recognising a C-terminal region of STE12 involved in transcriptional  
activation, wherein said recognised region or domain is comprised by  
the polypeptide having the amino acid sequence as shown in SEQ ID  
NO:8; and/or
- 10                   c) is competing with a polypeptide having the amino acid sequence as  
shown in SEQ ID NO:8 for binding to a predetermined binding part-  
ner, including a polynucleotide having an affinity for said polypeptide.
- 15                   v) a polynucleotide, the complementary strand of which hybridizes, under  
stringent conditions, with a polynucleotide as defined in any of (i), (ii), (iii),  
and (iv), said polynucleotide encoding a polypeptide having the amino acid  
sequence as shown in SEQ ID NO:8, or a fragment thereof, wherein said  
fragment
- 20                   a) has *Mucor racemosus* STE12 activity and is a regulator of morphol-  
ogy of a dimorphic fungal cell; and/or
- 25                   b) is recognised by an antibody, or a binding fragment thereof, which is  
i) capable of recognising an N-terminal region of STE12 involved in  
polynucleotide binding and/or ii) capable of recognising an induction  
domain located in the central region of STE12 and/or iii) capable of  
recognising a C-terminal region of STE12 involved in transcriptional  
activation, wherein said recognised region or domain is comprised by  
the polypeptide having the amino acid sequence as shown in SEQ ID  
30                   NO:8; and/or
- 35                   c) is competing with a polypeptide having the amino acid sequence as  
shown in SEQ ID NO:8 for binding to a predetermined binding part-  
ner, including a polynucleotide having an affinity for said polypeptide,  
and

- vi) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iv) and (v),

5

and the complementary strand of such a polynucleotide.

Accordingly, the first nucleotide sequence in one preferred embodiment preferably comprises nucleotides 1 to 384 of SEQ ID NO:7.

10

In another embodiment, the first nucleotide sequence comprises or essentially consists of the coding sequence of *ste12* encoding a transcription factor of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14064; and.

15

In yet another embodiment, the first nucleotide sequence encodes a polypeptide having the amino acid sequence as shown in SEQ ID NO:8.

In a further embodiment, the first nucleotide sequence encodes a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:8, wherein said fragment

20

- a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

25

- b) is recognised by an antibody, or a binding fragment thereof, which is  
i) capable of recognising an N-terminal region of STE12 involved in polynucleotide binding and/or ii) capable of recognising an induction domain located in the central region of STE12 and/or iii) capable of recognising a C-terminal region of STE12 involved in transcriptional  
activation, wherein said recognised region or domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:8; and/or

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- c) is competing with a polypeptide having the amino acid sequence as shown in SEQ ID NO:8 for binding to a predetermined binding partner, including a polynucleotide having an affinity for said polypeptide.

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In a still further embodiment, the first polynucleotide sequence comprises a polynucleotide the complementary strand of which hybridizes under stringent conditions with a polypeptide that

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a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

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b) is recognised by an antibody, or a binding fragment thereof, which is  
i) capable of recognising an N-terminal region of STE12 involved in polynucleotide binding and/or ii) capable of recognising an induction domain located in the central region of STE12 and/or iii) capable of recognising a C-terminal region of STE12 involved in transcriptional activation, wherein said recognised region or domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:8; and/or

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c) is competing with a polypeptide having the amino acid sequence as shown in SEQ ID NO:8 for binding to a predetermined binding partner, including a polynucleotide having an affinity for said polypeptide.

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There is also provided a polynucleotide comprising a first nucleotide sequence which is degenerate to any of the sequences described herein immediately above, as well as a polynucleotide comprising a first nucleotide sequence in the form of the the complementary strand of polynucleotides described herein immediately above.

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The STE12 transcription factor consists of a N-terminal region involved in DNA binding (~70 % homology between fungal homologues in this region), an induction domain located in the central region of the protein and a C-terminal region that is involved in transcriptional activation.

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In yeast, STE12 binds to the pheromone-responsive element found in the upstream region of many genes inducible by the mating pheromone  $\alpha$ - and a-factor (Pi et al., 1997: Transcriptional activation upon pheromone stimulation mediated by a small domain of *S. cerevisiae* Ste12p. *Mol Cel Biol* 17: 6410-6418). A conserved 6 aa sequence (induction domain) is found among cloned fungal STE12 homologues

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(position 305 to 310 in the yeast protein). The DNA binding region shows at least two aa stretches which are highly conserved (FFLATA and TQKKQKVF; Yue et al. 1999; Genetics 153: 1601-1615: The STE12a homolog is required for haploid filamentation but largely dispensable for mating and virulence in *Cryptococcus neoformans*).

The STE12 'conserved' structural motif (only four STE12 homologues are cloned to date including the *Mucor racemosus* counterpart) is shown below. In one preferred embodiment, such a conserved structural motif is recognised by the antibody used to define fragments of STE12 in accordance with the present invention.

Length = 111  
Score = 140 bits (352), Expect = 5e-35

Mucor: 43 ISCVLWNDLFFITGTDIVRSLTFRHAFGRPVTNAKKFEEGIFSDLRNLPKPGHDARLEEP 102  
Sbjct: 16 VSCVYWNNLYFITGTDIVRCIVYKFEHFGRKIIDRKKFEEGIFSDLRNLCGADAILEPP 75

Mucor: 103 KSELLDMLYKNNCIRTQKKQKVFFWF 128  
Sbjct: 76 RSEFLEFLFKNSCLRTQKKQKVFFWF 101

#### Expression signals capable of being regulated during growth of a dimorphic fungal cell

Expression signals as used herein preferably comprise a promoter element including a promoter sequence. As used herein, a promoter sequence is a DNA sequence which is recognized by the particular filamentous fungus for expression purposes. It is operably linked to a DNA sequence encoding the above defined polypeptides. Such linkage comprises positioning of the promoter with respect to the initiation codon of the DNA sequence encoding the signal sequence of the disclosed transformation vectors. The promoter sequence contains transcription and translation control sequences which mediate the expression of the signal sequence and heterologous polypeptide.

The promoter may be any DNA sequence that shows strong and/or regulated transcriptional activity in these species, and may be derived from genes encoding both extracellular and intracellular proteins, such as glucoamylases and glycolytic enzymes. The promoter may be either a heterologous promoter or a homologous promoter, *i.e.*, the promoter for a gene that is either non-native or native, respec-



tively, to the host strain being used. Useful promoters according to the present invention are e.g. the *gpd1* promoter and the *prnC* promoter from *M. racemosus*.

5 *gpd1* encodes glyceraldehyde-3-phosphate dehydrogenase (GPD) in *Mucor racemosus*, and transcription of *gpd1* is detectable during vegetative growth under both aerobic and anaerobic conditions. The transcription of *gpd1* in *M. racemosus* is significantly higher on fermentable carbon sources than on non-fermentable carbon sources during growth under aerobic conditions, indicating that *gpd1* expression is subjected to carbon catabolite regulation. A direct correlation can be established  
10 between the abundance of *gpd1* mRNA and the concentration of sugar in the medium during growth.

The promoter sequence may in one embodiment be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the promoter  
15 sequence with the gene of choice or with a selected signal peptide. Terminators and polyadenylation sequences may also be derived from the same sources as the promoters. Enhancer and regulatory sequences may also be inserted into the construct.

20 The promoters and enhancers that control the transcription of protein-encoding genes are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

25 The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp  
30 of DNA, and containing one or more recognition sites for transcriptional activator proteins. At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box a discrete element overlying the start site itself helps to fix the place of initiation.

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Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities. They have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

In one preferred embodiment of the present invention there is provided a polynucleotide comprising

- i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto

- ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

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wherein the first and second nucleotide sequences are not natively associated, and

wherein the second nucleotide sequence comprises an expression signal comprising at least one element of a promoter region capable of being regulated, during growth of a dimorphic fungal cell, by any one or more factors including

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- a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,

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- b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,

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- c) the growth phase of the dimorphic fungal cell, and

- d) the growth rate of the dimorphic fungal cell.

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The regulation is preferably an induction or a repression, including an induction or a repression of the expression of the first nucleotide sequence being operably linked to the expression signal, as compared to a predetermined expression level, by at least a factor of 1.02, such as at least a factor 1.05, for example at least a factor 1.10, such as at least a factor 1.15, for example at least a factor 1.20, such as at least a factor 1.25, for example at least a factor 1.30, such as at least a factor 1.35, for example at least a factor 1.40, such as at least a factor 1.45, for example at least a factor 1.50, such as at least a factor 1.75, for example at least a factor 2.0, such as at least a factor 2.5, for example at least a factor 5.0, such as at least a factor 7.5, for example at least a factor 10, such as at least a factor 15, for example at

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least a factor 20, such as at least a factor 30, for example at least a factor 40, such as at least a factor 50, for example at least a factor 75, such as at least a factor 100, for example at least a factor 150, such as at least a factor 200, for example at least a factor 500, such as at least a factor 1000, for example at least a factor 2000, such as at least a factor 4000, for example at least a factor 8000, such as at least a factor 10000, for example at least a factor 15000, such as at least a factor 25000, for example at least a factor 50000, such as at least a factor 75000, for example at least a factor 100000.

10 Predetermined expression level as used herein signifies any expression level detectable by means of any assay including any determination or analysis of mRNA and/or polypeptide production prior to any change in any one or more of the above-mentioned factors.

15 Northern blots are used to measure e.g. induction of expression using a Cyclone Storage Phosphor System (Packard) and the OptiQuant image analysis software. A linear dynamic range of 5 orders of magnitude with only a 5% standard deviation is possible with the above apparatus. Dilution series of the RNA preparations (typically 20, 10 and 5  $\mu$ g total RNA per sample) can be used to estimate induction levels.

20 Such a change in e.g. mRNA following e.g. induced gene expression may either result from an addition to the growth medium of the factor, said addition resulting in an increased or essentially constant concentration of said factor in the growth medium, or a removal from the growth medium of the factor, said removal resulting in a decreased concentration, or an essentially constant concentration, of said factor

25 in the growth medium.

The addition may be an addition from an external source, or it may be an addition to the growth medium of a factor produced or consumed by the microbial cell in question, including a fungal cell, including a dimorphic fungal cell.

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The removal from the growth medium of factor may be a selective removal, e.g. a filtration or precipitation of factor, resulting in a decreased concentration, or an essentially constant concentration, of said factor being present in the growth medium. Alternatively, the removal may be generated by consumption of the factor

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by the microbial cell in question, including a fungal cell, including a dimorphic fungal cell.

5 Accordingly, it is possible to determine - at a first predetermined timepoint during the cultivation of the microbial cell, including a fungal cell, including a dimorphic fungal cell - the predetermined level of expression of the first nucleotide sequence being operably linked to the expression signal, and to determine for that same timepoint, the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and 10 the presence or absence of a cell constituent, or a precursor thereof.

Concomitant determinations at a later and second predetermined timepoint of i) the expression of the first nucleotide sequence, and ii) the relative change of the growth 15 composition as compared to the growth composition at the first predetermined timepoint allows the skilled person to evaluate the effect of a) the change of the growth composition on b) the expression of the first nucleotide sequence operably linked to the expression signal.

20 This evaluation allows the skilled person to conclude whether the expression of the first nucleotide sequence encoding the at least one regulator of morphology is induced or repressed by the changed growth conditions. The skilled person will in one embodiment change only one factor at any one time and maintain the remaining factors at an essentially unchanged status or level as far as this is possible. In other 25 embodiments, more than one factor is changed simultaneously, or sequentially in any order.

The change in the composition of the growth medium may lead to either an increased or a decreased expression of the first nucleotide sequence encoding the at 30 least one regulator of morphology, said increased or decreased expression resulting - directly or indirectly - in an increased or a decreased production of regulator of morphology, respectively, said increased or decreased production of regulator results in an improved filamentation of a fungal cell, including a dimorphic fungal cell, or in a dimorphic shift of a dimorphic fungal cell. The dimorphic shift of the 35 dimorphic fungal cell preferably also results in an improved filamentation.

It is understood that the increased or a decreased expression of the first nucleotide sequence results from the expression signal comprised in the second nucleotide sequence being regulatable by the change in the composition of the growth medium.

5

When the second nucleotide sequence comprising an expression signal is regulatable by at least one factor of a growth medium composition:

the carbon source is preferably e.g., starch, cellulose, pectin, oligosaccharides, glucose, galactose, glycerol, ethanol, and any change in the composition or amount of the carbon source regulate in one embodiment the expression signal comprised by the second nucleotide sequence,

the nitrogen source is preferably proteins, peptides (like casaminoacids), amino acids, including any composition of naturally occurring amino acids as listed herein below,

Amino Acids	Codons
Alanine Ala	A
Cysteine Cys	C
Aspartic acid Asp	D
Glutamic acid Glu	E
Phenylalanine Phe	F
Glycine Gly	G
Histidine His	H
Isoleucine Ile	I
Lysine Lys	K
Leucine Leu	L
Methionine Met	M
Asparagine Asn	N
Proline Pro	P
Glutamine Gln	Q
Arginine Arg	R
Serine Ser	S
Threonine Thr	T
Valine Val	V
GCA GCC GCG GCU	
UGC UGU	
GAC GAU	
GAA GAG	
UUC UUU	
GGA GGC GGG GGU	
CAC CAU	
AUA AUC AUU	
AAA AAG	
UUA UUG CUA CUC CUG CUU	
AUG	
AAC AAU	
CCA CCC CCG CCU	
CAA CAG	
AGA AGG CGA CGC CGG CGU	
AGC AGU UCA UCC UCG UCU	
ACA ACC ACG ACU	
GUA GUC GUG GUU	

Tryptophan Trp	W	UGG
Tyrosine Tyr	Y	UAC UAU

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5 and precursors and/or derivatives thereof, like citrulline, ornithine, and the like, as well as inorganic salts (like ammonium sulfate, acetamide, nitrates or nitrites), and any change in the composition or amount of the nitrogen source regulate in one embodiment the expression signal comprised by the second nucleotide sequence,

10 the oxygen content is preferably one characterised as sufficient to result in aerobic growth, or an oxygen content sufficiently low to qualify growth conditions characterised as anaerobic growth. Anaerobic is per definition an atmosphere with no oxygen. *Mucor racemosus* adopts a filamentous morphology when levels of oxygen are above 1.2 micromolar. At lower levels *Mucor racemosus* grows with a unicellular,

15 essentially spherical morphology provided that a fermentable hexose and organic nitrogen are present. However, *Mucor racemosus* can grow as filaments in lower concentrations of oxygen (also complete anaerobic atmosphere) in case of physiological stress. Also the opposite situation is observed: aerobic yeast growth may occur under specific growth conditions (e.g., high glucose

20 concentration after the shift from anaerobic growth to aerobic growth). In one embodiment, changes in oxygen levels regulate the expression signal comprised by the second nucleotide sequence,

the ionic strength, including NaCl content, is preferably that of a standard growth

25 medium, and changes therein regulate the expression signal comprised by the second nucleotide sequence in one embodiment of the present invention,

the pH is preferably within the range of physiological pH values, and changes therein regulate the expression signal comprised by the second nucleotide sequence in one embodiment,

30 low molecular weight compounds are preferably salts (sulfate, phosphate, nitrate), and/or metals (e.g., copper), and changes therein regulate the expression signal comprised by the second nucleotide sequence in one embodiment,

cAMP levels are preferably those sufficient for inducing an improved filamentation and/or a dimorphic shift. Mucor species having a unicellular, essentially spherical morphology may have an intracellular cAMP level of about 2-10 mM intracellular cAMP, while filamentous growing Mucor species generally have lower levels of cAMP. When added exogenously, about 40 mM dbcAMP preferably induces a dimorphic shift from aerobic, Mucor filamentous growth to aerobic, Mucor unicellular, essentially spherical morphology. Lower concentrations such as e.g. 10 mM dbcAMP may also work for some species. In one embodiment, changes in cAMP levels regulate the expression signal comprised by the second nucleotide sequence,

presence or absence of a cell constituent, or a precursor thereof, is preferably a cofactor, a vitamin, or a lipid, and the like, and in one embodiment, changes in cell constituent levels regulate the expression signal comprised by the second nucleotide sequence.

It is preferred that the second polynucleotide comprises at least one element of a promoter region comprised by the expression signal, preferably a promoter element regulated, during growth of the dimorphic fungal cell, by the carbon source of the growth medium and/or the oxygen content and the carbon source of the growth medium. It is preferred that the at least one element of the promoter region is induced by the presence in the growth medium, or the addition to the growth medium, of a carbon source. The carbon source preferably comprises a hexose, preferably glucose and/or galactose.

#### Promoter elements of second nucleotide sequences

In one embodiment, the present invention relates to an expression signal comprising a promoter element isolated from a Mucor racemosus glyceraldehyde-3-phosphate dehydrogenase promoter, preferably gpd1P, including, but not limited to, a promoter element comprising nucleotides 1 to 741 of SEQ ID NO:9, or a fragment thereof capable of directing gene expression in a fungal host cell.

Accordingly, in one embodiment of the present invention at least one element of the promoter region comprised by the expression signal is selected from the group consisting of



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- 10
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- i) a polynucleotide comprising nucleotides 1 to 741 of SEQ ID NO:9,
  - ii) a polynucleotide comprising or essentially consisting of the promoter region of *gpd1* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14066; and
  - iii) a polynucleotide comprising at least one fragment of SEQ ID NO:9, wherein said fragment
    - a) is capable of directing gene expression in a dimorphic fungal cell; and/or
    - b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 741 of SEQ ID NO:9; and/or
    - c) is capable of being regulated, during growth of the dimorphic fungal cell, by one or more of
      - the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,
      - the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
      - the growth phase of the dimorphic fungal cell, and
      - the growth rate of the dimorphic fungal cell, and

iv) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide

5 a) is capable of directing gene expression in a dimorphic fungal cell; and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 741 of SEQ ID NO:9,

10

and the complementary strand of such a polynucleotide.

The expression signal comprising a promoter element isolated from a *Mucor racemosus* glyceraldehyde-3-phosphate dehydrogenase promoter, preferably gpd1P, is in one embodiment also capable of directing gene expression in other fungal cells including fungal cells that are not dimorphic fungal cells. At least one promoter element of gpd1P is induced by the carbon source of the growth medium, said induction resulting in an increased expression of the regulator of morphology being encoded by a nucleotide sequence operably linked to said promoter element.

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In one embodiment, the at least one element of the promoter region comprised by the expression signal comprises nucleotides 1 to 741 of SEQ ID NO:9, or a fragment thereof, wherein said fragment is capable of directing gene expression in a fungal cell, including a dimorphic fungal cell, and is regulatable, during growth of the fungal cell, including a dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 741 of SEQ ID NO:9. The factor is preferably selected from the group consisting of

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a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,

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b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,

5 c) the growth phase of the dimorphic fungal cell, and

d) the growth rate of the dimorphic fungal cell.

Also, the at least one element of the promoter region comprised by the expression  
10 signal preferably comprises nucleotides 1 to 741 of SEQ ID NO: 9, or the promoter region of *gpd1* of *M. racemosus*, as deposited with DSMZ under accession number DSM 14066.

In another embodiment, the present invention relates to an expression signal  
15 comprising a promoter element isolated from the promoter region of *Mucor racemosus prnC*, including, but not limited to, a promoter element comprising nucleotides 1 to 755 of SEQ ID NO:10, or a fragment thereof capable of directing gene expression in a fungal host cell.

20 Accordingly, in one embodiment of the present invention, the at least one element of the promoter region comprised by the expression signal is preferably selected from the group consisting of

25 i) a polynucleotide comprising nucleotides 1 to 755 of SEQ ID NO:10,

ii) a polynucleotide comprising or essentially consisting of the promoter region of *prnC* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14067; and

30 iii) a polynucleotide comprising at least one fragment of SEQ ID NO:10, wherein said fragment

a) is capable of directing gene expression in a dimorphic fungal cell;  
and/or

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- b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 755 of SEQ ID NO:10; and/or
- 5 c) is capable of being regulated, during growth of the dimorphic fungal cell, by one or more of
- 10      ◦ the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof
  - 15      ◦ the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
  - 20      ◦ the growth phase of the dimorphic fungal cell, and
  - 25      ◦ the growth rate of the dimorphic fungal cell, and
- iv) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide
- 25 a) is capable of directing gene expression in a dimorphic fungal cell; and/or
- 30 b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 755 of SEQ ID NO:10,
- and the complementary strand of such a polynucleotide.

5 The expression signal comprising a promoter element isolated from *Mucor racemosus* *pmC* is in one embodiment also capable of directing gene expression in other fungal cells including fungal cells that are not dimorphic fungal cells. At least one promoter element of *pmC* is induced by aerobicity during filamentous growth of a fungal cell, including a dimorphic fungal cell, said induction resulting in an increased expression of the regulator of morphology being encoded by a nucleotide sequence operably linked to said promoter element.

10 The at least one element of the promoter region comprised by the expression signal preferably comprises nucleotides 1 to 755 of SEQ ID NO:10, or a fragment thereof, wherein said fragment is capable of directing gene expression in a fungal cell, including a dimorphic fungal cell, and is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 755 of SEQ ID NO:10. The factor is preferably selected from the group consisting of

- 20 a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,
- 25 b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
- c) the growth phase of the dimorphic fungal cell, and
- 30 d) the growth rate of the dimorphic fungal cell.

35 In another preferred embodiment, the at least one element of the promoter region comprised by the expression signal comprises nucleotides 1 to 755 of SEQ ID NO: 10, or the promoter region of *pmC* of *M. racemosus*, as deposited with DSMZ under accession number DSM 14067.

In a further embodiment, there is provided a polynucleotide according to the invention and operably linked to a further polynucleotide selected from the group of polynucleotides consisting of a 3' untranslated region, or a fragment thereof, and/or a 5' upstream region, or a fragment thereof.

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#### Regulators produced from expression of first nucleotide sequences

The regulator of morphology is in one embodiment a polypeptide capable of regulating gene transcription in a dimorphic fungal cell by forming an interaction with a recognition motif of a promoter region having an affinity for the regulator of morphology. In another embodiment the regulator of morphology comprises a kinase activity, or comprises a regulator of a kinase activity, or is regulated by a kinase activity.

In one embodiment, the expression of the first nucleotide sequence directed by the second nucleotide sequence results in the production of the regulator of morphology, and said production results in the dimorphic fungal cell adopting a unicellular, essentially spherical morphology. In the absence of production of the regulator of morphology, the cell adopts a filamentous morphology and grows in the form of a mycelium having a filamentous structure and comprising multinucleated or uninucleated cells. When the cell is a zygomycete, the individual cells are multinucleated. When the cell is e.g. *Candida* or *Arxula*, the individual cells are uninucleated.

Accordingly, in an embodiment wherein the production of the regulator of morphology results in the dimorphic fungal cell adopting a unicellular, essentially spherical morphology, and wherein the absence of production of the regulator of morphology results in the dimorphic fungal cell adopting a filamentous morphology, the present invention relates to a regulatably reduced production of such a regulator of morphology.

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In the above-mentioned embodiment wherein the invention relates to a repressor capable of repression of a dimorphic shift, or capable of repression of filamentation, or capable of repression of an improved filamentation in a dimorphic fungal cell, the lifting of the repression (de-repression), by means of reducing or eliminating the expression of the first nucleotide sequence encoding the repressor, preferably

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results in a dimorphic shift and/or an improved filamentation of the dimorphic fungal cell.

5 In order to further promote a dimorphic shift, or in order to further improve the filamentation of the dimorphic fungal cell, the cell may comprise a further regulator of morphology encoded by a nucleotide sequence not natively associated with said further regulator of morphology, wherein the production of said further regulator of morphology is positively correlated with a dimorphic shift and/or an improved filamentation of the dimorphic fungal cell. The further regulator of morphology in one  
10 embodiment is an activator, the production of which results in a dimorphic shift and/or an improved filamentation of the dimorphic fungal cell.

An activator may be present in the dimorphic cell irrespective of whether the above-mentioned repressor is present or not. It will be understood that both the repressor  
15 and the activator are encoded by a nucleotide sequence operably linked to a regulatable promoter not natively associated therewith, including a second nucleotide sequence.

Accordingly, in a further embodiment, the invention relates to a regulator of morphology in the form of an activator, the expression of which results in a dimorphic shift, or results in filamentation, or results in an improved filamentation in a dimorphic fungal cell, said activator being encoded by a nucleotide sequence operably linked to a regulatable promoter not natively associated therewith, including a  
20 second nucleotide sequence.

25 In one embodiment, the activator is not expressed, or expressed at a reduced level, when the microbial cell, including a fungal cell, including a dimorphic fungal cell, adopts a unicellular, essentially spherical morphology. Following induction, increased expression or otherwise, the production of the activator results in the cell  
30 adopting a filamentous morphology and growing in the form of a mycelium having a filamentous structure and comprising multinucleated or uninucleated cells. When the cell is a zygomycete, the individual cells are multinucleated. When the cell is e.g. Geotrichum, Candida or Arxula, the individual cells are uninucleated.

In the above-mentioned embodiment wherein the invention relates to activator, the induction of the activation, by means of increasing the expression of the first nucleotide sequence encoding the activator, preferably results in a dimorphic shift and/or an improved filamentation of the dimorphic fungal cell.

5

In order to further promote a dimorphic shift, or in order to further improve the filamentation of the dimorphic fungal cell, the cell may comprise a further activator encoded by a nucleotide sequence not natively associated with said further activator, wherein the production of said further activator results in a dimorphic shift and/or

10

When the regulator of morphology directs a dimorphic shift of a dimorphic fungal cell, the shift is in one embodiment a shift from a first morphological condition of the dimorphic fungal cell, wherein the fungal cell has an unicellular, essentially spherical morphology, to a second morphological condition of the dimorphic fungal cell, wherein the fungal cell is characterised by a mycelium capable of filamentous growth.

15

In another embodiment, the dimorphic shift of the dimorphic fungal cell is a shift from a second morphological condition of the dimorphic fungal cell, wherein the fungal cell is characterised by a mycelium capable of filamentous growth, to a first morphological condition of the dimorphic fungal cell, wherein the fungal cell has an unicellular, essentially spherical morphology.

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In a further embodiment, the first morphological condition, wherein the fungal cell has an unicellular, essentially spherical morphology, is further characterised by an essentially isodiametrical or spherical shape of the fungal cells, or by an essentially non-polarised growth of the cells. The second morphological condition, characterised by filamentous growth, is further characterised by an essentially elongated, hyphal cell shape resulting from a polarised growth of a fungal cell characterised by the first morphological condition.

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Although a dimorphic shift can only be observed for a dimorphic fungal cell, an improved filamentation can result from the expression of a regulator of morphology



in any microbial cell capable of displaying a filamentous morphology, including any filamentous fungal cell, including any zygomycete.

Accordingly, there is provided in one embodiment, a nucleotide sequence encoding a regulator of morphology, wherein an increased or decreased expression of said regulator results in an improved filamentation of a fungal cell without inducing concomitantly therewith a dimorphic shift when the cell is a dimorphic fungal cell, including a dimorphic fungal cell, the morphology of which is regulatable by the regulator of morphology and wherein the cell is capable of growing as a multinucleated cell having a unicellular, essentially spherical morphology and/or capable of growing as a mycelium having a filamentous structure and comprising multinucleated cells, including dimorphic fungal cells comprising multinucleated and multipolar cells, and dimorphic fungal cells that are multinucleated as well as bipolar or monopolar. In one embodiment, the dimorphic fungal cell belongs to the class of Zygomycetes, including the order of Mucorales, including from the order of Mucorales a genus preferably selected from the group of genera consisting of Mucor, Thermomucor, Rhizomucor, Mycotypha, Rhizopus, and Cokeromyces, including Cokeromyces recurvatus. Preferred Mucor species according to this embodiment of the invention are those mentioned herein above, including M. racemosus, M. hiemalis, M. rouxii, M. genevensis, M. bacilliformis, and M. subtilissimus. M. racemosus is particularly preferred.

In a still further embodiment, there is provided a nucleotide sequence encoding a regulator of morphology, wherein an increased or decreased expression of said regulator results in an improved filamentation of a fungal cell that is not a dimorphic cell. Non-limiting examples of such cells are listed herein above.

In one preferred embodiment, the expression of said first polynucleotide results in the production of the regulator in an increased or decreased amount, as compared to the amount of regulator produced, when the first polynucleotide encoding the regulator is operably linked to the native promoter region under substantially identical growth conditions, and wherein the expression of said first polynucleotide and the production of the regulator in an increased or a decreased amount results in an improved filamentation, or in a dimorphic shift of the dimorphic fungal cell. Substantially identical growth conditions are conditions characterised by deviations

of growth composition factors within a 5 percent range, including deviations within a range characterised by standard deviations of measurement.

5 Preferably, an increased amount of the regulator of morphology is produced from the expression of the first nucleotide sequence encoding said regulator. However, the invention also relates to an embodiment, wherein a decreased amount of the regulator of morphology is produced from the expression of the first nucleotide sequence encoding said regulator.

10 The amount of regulator produced is preferably increased or decreased at least by a factor of 1.02, such as at least by a factor 1.05, for example at least by a factor 1.10, such as at least a factor 1.15, for example at least by a factor 1.20, such as at least a factor 1.25, for example at least by a factor 1.30, such as at least a factor 1.35, for example at least a factor 1.40, such as at least a factor 1.45, for example, at least by  
15 a factor 1.50, such as at least by a factor 1.75, for example at least a factor 2.0, such as at least a factor 2.5, for example at least a factor 5.0, such as at least a factor 7.5, for example at least a factor 10, such as at least a factor 15, for example at least a factor 20, such as at least a factor 30, for example at least by a factor 40, such as at least a factor 50, for example at least by a factor 75, such as at least a  
20 factor 100, for example at least a factor 150, such as at least a factor 200, for example at least a factor 500, such as at least 1000, for example at least 5000, such as at least 10000, such as at least 20000, for example at least 40000, such as at least 60000, such as at least 80000, for example at least 100000.

## 25 Regulators of signal transduction pathways dependent on cAMP and MAP-kinase

In one embodiment, a regulator of morphology is related to a polypeptide encoded by a first polynucleotide comprising a first polynucleotide sequence forming part of a cAMP-dependent signal transduction pathway of a microbial cell, including a fungal  
30 cell, including a *Mucor* species, including *M. racemosus*,

wherein the expression of said first polynucleotide results in the production of the regulator in an increased or decreased amount, as compared to the amount of regulator produced, when the first polynucleotide encoding the regulator is

operably linked to the native promoter region under substantially identical growth conditions,

5 wherein the expression of said first polynucleotide and the production of the regulator in an increased or a decreased amount results in an improved filamentation, or in a dimorphic shift of the dimorphic fungal cell.

10 In another embodiment, the regulator of morphology is preferably a polypeptide encoded by a first polynucleotide comprising a first polynucleotide sequence forming part of a MAP kinase-dependent signal transduction pathway of a microbial cell, including a fungal cell, including a Mucor species, including *M. racemosus*,

15 wherein the expression of said first polynucleotide results in the production of the regulator in an increased or decreased amount, as compared to the amount of regulator produced, when the first polynucleotide encoding the regulator is operably linked to the native promoter region under substantially identical growth conditions,

20 wherein the expression of said first polynucleotide and the production of the regulator in an increased or a decreased amount results in an improved filamentation, or in a dimorphic shift of the dimorphic fungal cell.

25 In one preferred embodiment, there is provided an isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:2, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 2, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide

- 30 a) has Mucor racemosus protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a cAMP binding domain of Mucor racemosus protein kinase A, wherein said cAMP binding domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID
- 35 NO:2; and/or

- c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the catalytic subunit for protein kinase A.

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In another preferred embodiment there is provided an isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:4, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 4, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide

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- a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

15

- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or

20

- c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.

25

In yet another embodiment there is provided an isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:6, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 6, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide

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- a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

35

- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mi-

togen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or

- 5 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.

10 In a still further embodiment, there is provided an isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:8, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 8, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide

- 15 a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

- b) is recognised by an antibody, or a binding fragment thereof, which is  
 20 i) capable of recognising an N-terminal region of STE12 involved in polynucleotide binding and/or ii) capable of recognising an induction domain located in the central region of STE12 and/or iii) capable of recognising a C-terminal region of STE12 involved in transcriptional activation, wherein said recognised region or domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID  
 25 NO:8; and/or

- c) is competing with a polypeptide having the amino acid sequence as shown in SEQ ID NO:8 for binding to a predetermined binding partner, including a polynucleotide having an affinity for said polypeptide.

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Functional equivalents and variants of polynucleotides encoding a regulator of morphology and polypeptides comprising such a regulator

35 As certain amino acids may be substituted for other amino acids in a polypeptide structure without appreciable loss of interactive binding capacity, and as it is the

interactive capacity and nature of a polypeptide that defines the biological activity of the polypeptide, certain amino acid sequence substitutions can be made in a polypeptide sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a polypeptide with functionally equivalent properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

Functional equivalents and variants are used interchangeably herein. In one preferred embodiment of the invention there is also provided variants of a regulator of morphology, and variants of fragments thereof.

The regulator polypeptide in one embodiment is preferably one, wherein a substantially identical morphological shift is obtained from the production in a dimorphic fungal cell, under substantially identical conditions, of substantially identically amounts of i) the polypeptide comprising the regulator of morphology of a dimorphic fungal cell, and ii) a functionally equivalent polypeptide comprising a functionally equivalent regulator of morphology, including any fragments thereof. A functionally equivalent polypeptide, or a fragment thereof, preferably comprises at least one conservative amino acid substitution. However, the invention is not limited to functional equivalents in the form of regulators comprising conservative substitutions.

When being polypeptides, variants are determined on the basis of their degree of identity or their homology with a predetermined amino acid sequence, said predetermined amino acid sequence being one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8, respectively, or, when the variant is a fragment, a fragment of any of the aforementioned amino acid sequences, respectively.

Accordingly, variants preferably have at least 75% sequence identity, for example at least 80% sequence identity, such as at least 85 % sequence identity, for example at least 90 % sequence identity, such as at least 91 % sequence identity, for example at least 91% sequence identity; such as at least 92 % sequence identity, for example at least 93 % sequence identity, such as at least 94 % sequence identity, for example at least 95 % sequence identity, such as at least 96 %

sequence identity, for example at least 97% sequence identity, such as at least 98 % sequence identity, for example 99% sequence identity with the predetermined sequence.

- 5 Sequence identity is determined in one embodiment by utilising fragments of regulator peptides comprising at least 25 contiguous amino acids and having an amino acid sequence which is at least 80%, such as 85%, for example 90%, such as 95%, for example 99% identical to the amino acid sequence of any of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, and SEQ ID NO: 8, respectively, wherein the  
10 percent identity is determined with the algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

- The following terms are used to describe the sequence relationships between two or more polynucleotides: "predetermined sequence", "comparison window", "sequence  
15 identity", "percentage of sequence identity", and "substantial identity".

- A "predetermined sequence" is a defined sequence used as a basis for a sequence comparison; a predetermined sequence may be a subset of a larger sequence, for example, as a segment of a full-length DNA or gene sequence given in a sequence  
20 listing, such as a polynucleotide sequence of SEQ ID NO:1, or may comprise a complete DNA or gene sequence. Generally, a predetermined sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length.

- 25 Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a  
30 "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a predetermined sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may  
35 comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the

predetermined sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

- Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected.
- The homology between amino acid sequences may also be calculated using well known algorithms such as any one of BLOSUM 30, BLOSUM 40, BLOSUM 45, BLOSUM 50, BLOSUM 55, BLOSUM 60, BLOSUM 62, BLOSUM 65, BLOSUM 70, BLOSUM 75, BLOSUM 80, BLOSUM 85, and BLOSUM 90.
- The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a predetermined sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the predetermined sequence to the polynucleotide sequence which may include deletions or additions



which total 20 percent or less of the predetermined sequence over the window of comparison. The predetermined sequence may be a subset of a larger sequence, for example, as a segment of the full-length SEQ ID NO:1 polynucleotide sequence illustrated herein.

5

As applied to polypeptides, a degree of identity of amino acid sequences is a function of the number of identical amino acids at positions shared by the amino acid sequences. A degree of homology or similarity of amino acid sequences is a function of the number of amino acids, i.e. structurally related, at positions shared by the amino acid sequences.

10

An "unrelated" or "non-homologous" sequence shares less than 40% identity, though preferably less than 25% identity, with one of the regulator polypeptide sequences of the present invention. The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

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20

Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine, a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

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Additionally, variants are also determined based on a predetermined number of conservative amino acid substitutions as defined herein below. Conservative amino acid substitution as used herein relates to the substitution of one amino acid (within

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a predetermined group of amino acids) for another amino acid (within the same group), wherein the amino acids exhibit similar or substantially similar characteristics.

5 Within the meaning of the term "conservative amino acid substitution" as applied herein, one amino acid may be substituted for another within the groups of amino acids indicated herein below:

- 10 i) Amino acids having polar side chains (Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, Tyr, and Cys,)
- ii) Amino acids having non-polar side chains (Gly, Ala, Val, Leu, Ile, Phe, Trp, Pro, and Met)
- 15 iii) Amino acids having aliphatic side chains (Gly, Ala Val, Leu, Ile)
- iv) Amino acids having cyclic side chains (Phe, Tyr, Trp, His, Pro)
- v) Amino acids having aromatic side chains (Phe, Tyr, Trp)
- 20 vi) Amino acids having acidic side chains (Asp, Glu)
- vii) Amino acids having basic side chains (Lys, Arg, His)
- 25 viii) Amino acids having amide side chains (Asn, Gln)
- ix) Amino acids having hydroxy side chains (Ser, Thr)
- x) Amino acids having sulphur-containing side chains (Cys, Met),
- 30 xi) Neutral, weakly hydrophobic amino acids (Pro, Ala, Gly, Ser, Thr)
- xii) Hydrophilic, acidic amino acids (Gln, Asn, Glu, Asp), and
- 35 xiii) Hydrophobic amino acids (Leu, Ile, Val)

Accordingly, a variant or a fragment thereof according to the invention may comprise, within the same variant of the sequence or fragments thereof, or among different variants of the sequence or fragments thereof, at least one substitution,  
5 such as a plurality of substitutions introduced independently of one another.

It is clear from the above outline that the same variant or fragment thereof may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

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The addition or deletion of at least one amino acid may be an addition or deletion of from preferably 2 to 250 amino acids, such as from 10 to 20 amino acids, for example from 20 to 30 amino acids, such as from 40 to 50 amino acids. However, additions or deletions of more than 50 amino acids, such as additions from 50 to 100  
15 amino acids, addition of 100 to 150 amino acids, addition of 150-250 amino acids, are also comprised within the present invention. The deletion and/or the addition may - independently of one another - be a deletion and/or an addition within a sequence and/or at the end of a sequence.

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The polypeptide fragments according to the present invention, including any functional equivalents thereof, may in one embodiment comprise less than 250 amino acid residues, such as less than 240 amino acid residues, for example less than 225 amino acid residues, such as less than 200 amino acid residues, for example less than 180 amino acid residues, such as less than 160 amino acid  
25 residues, for example less than 150 amino acid residues, such as less than 140 amino acid residues, for example less than 130 amino acid residues, such as less than 120 amino acid residues, for example less than 110 amino acid residues, such as less than 100 amino acid residues, for example less than 90 amino acid residues, such as less than 85 amino acid residues, for example less than 80 amino acid  
30 residues, such as less than 75 amino acid residues, for example less than 70 amino acid residues, such as less than 65 amino acid residues, for example less than 60 amino acid residues, such as less than 55 amino acid residues, for example less than 50 amino acid residues.

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique, well-known to those of skill in the art, further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy et al. (1994); Segal (1976); Prokop and Bajpai (1991); and Maniatis et al. (1982), each incorporated herein by reference, for that purpose.

The PCR-based strand overlap extension (SOE) for site-directed mutagenesis is particularly preferred for site-directed mutagenesis of the nucleic acid compositions of the present invention. The techniques of PCR are well-known to those of skill in the art.

In one embodiment, functional equivalents or variants of a regulator of morphology will be understood to exhibit amino acid sequences gradually differing from the preferred predetermined regulator sequence, as the number and scope of insertions, deletions and substitutions including conservative substitutions increases. This difference is measured as a reduction in homology between the preferred predetermined sequence and the fragment or functional equivalent.

Accordingly, all fragments or functional equivalents of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8 are included within the scope of this invention, regardless of the degree of homology that they show to the respective, predetermined regulator sequences disclosed herein. The reason for this is that some regions of the regulators are most likely readily mutable, or capable of being completely deleted, without any significant effect on the binding activity of the resulting fragment.

A functional variant obtained by substitution may well exhibit some form or degree of native regulator activity, and yet be less homologous, if residues containing functionally similar amino acid side chains are substituted. Functionally similar in this respect refers to dominant characteristics of the side chains such as hydrophobic, basic, neutral or acidic, or the presence or absence of steric bulk. Accordingly, in one embodiment of the invention, the degree of identity is not a principal measure of a fragment being a variant or functional equivalent of a preferred predetermined fragment according to the present invention.

Fragments sharing homology with fragments of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8, respectively, are to be considered as falling within the scope of the present invention when they are preferably at least about 90 percent homologous, for example at least 92 percent homologous, such as at least 94 percent homologous, for example at least 95 percent homologous, such as at least 96 percent homologous, for example at least 97 percent homologous, such as at least 98 percent homologous, for example at least 99 percent homologous with said predetermined fragment sequences, respectively. According to one embodiment of the invention the homology percentages refer to identity percentages.

Additional factors that may be taken into consideration when determining functional equivalence according to the meaning used herein are i) the ability of antisera to detect a functionally equivalent regulator of morphology according to the present invention, or ii) the ability of the functionally equivalent regulator of morphology to compete with a predetermined regulator of morphology in an assay.

In one embodiment, the sequence of immunogenically active amino acids within a known amino acid sequence is determined as described by Geysen in US 5,595,915

which is incorporated herein by reference. A further suitably adaptable method for determining structure and function relationships of peptide fragments is described by US 6,013,478, which is herein incorporated by reference. Also, methods of assaying the binding of an amino acid sequence to a receptor moiety are known to the skilled artisan.

In addition to conservative substitutions introduced into any position of a preferred predetermined regulator of morphology, or a fragment thereof, it may also be desirable to introduce non-conservative substitutions in any one or more positions of such a regulator.

A non-conservative substitution leading to the formation of a functionally equivalent fragment of regulator of morphology would for example i) differ substantially in polarity, for example a residue with a non-polar side chain (Ala, Leu, Pro, Trp, Val, Ile, Leu, Phe or Met) substituted for a residue with a polar side chain such as Gly, Ser, Thr, Cys, Tyr, Asn, or Gln or a charged amino acid such as Asp, Glu, Arg, or Lys, or substituting a charged or a polar residue for a non-polar one; and/or ii) differ substantially in its effect on polypeptide backbone orientation such as substitution of or for Pro or Gly by another residue; and/or iii) differ substantially in electric charge, for example substitution of a negatively charged residue such as Glu or Asp for a positively charged residue such as Lys, His or Arg (and vice versa); and/or iv) differ substantially in steric bulk, for example substitution of a bulky residue such as His, Trp, Phe or Tyr for one having a minor side chain, e.g. Ala, Gly or Ser (and vice versa).

Variants obtained by substitution of amino acids may in one preferred embodiment be made based upon the hydrophobicity and hydrophilicity values and the relative similarity of the amino acid side-chain substituents, including charge, size, and the like. Exemplary amino acid substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

The importance of the hydrophilic and hydrophobic amino acid indices in conferring interactive biologic function on a protein is well understood in the art (Kyte &

Doolittle, 1982 and Hopp, U.S. Pat. No. 4,554,101, each incorporated herein by reference).

It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant polypeptide, which in turn defines the interaction of the polypeptide with other molecules, for example, binding partners and polynucleotides,

Accordingly, in a further embodiment the present invention relates to functional variants comprising substituted amino acids having hydrophilic values or hydrophathic indices that are within  $\pm 4.9$ , for example within  $\pm 4.7$ , such as within  $\pm 4.5$ , for example within  $\pm 4.3$ , such as within  $\pm 4.1$ , for example within  $\pm 3.9$ , such as within  $\pm 3.7$ , for example within  $\pm 3.5$ , such as within  $\pm 3.3$ , for example within  $\pm 3.1$ , such as within  $\pm 2.9$ , for example within  $\pm 2.7$ , such as within  $\pm 2.5$ , for example within  $\pm 2.3$ , such as within  $\pm 2.1$ , for example within  $\pm 2.0$ , such as within  $\pm 1.8$ , for example within  $\pm 1.6$ , such as within  $\pm 1.5$ , for example within  $\pm 1.4$ , such as within  $\pm 1.3$  for example within  $\pm 1.2$ , such as within  $\pm 1.1$ , for example within  $\pm 1.0$ , such as within  $\pm 0.9$ , for example within  $\pm 0.8$ , such as within  $\pm 0.7$ , for example within  $\pm 0.6$ , such as within  $\pm 0.5$ , for example within  $\pm 0.4$ , such as within  $\pm 0.3$ , for example within  $\pm 0.25$ , such as within  $\pm 0.2$  of the value of the amino acid it has substituted.

The amino acid hydropathic index values as used herein are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5) (Kyte & Doolittle, 1982).

The amino acid hydrophilicity values are: arginine (+3.0); lysine (+3.0); aspartate (+3.0,  $\pm 0.1$ ); glutamate (+3.0,  $\pm 0.1$ ); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5,  $\pm 0.1$ ); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4) (U.S. 4,554,101).

Production of a desirable gene product by a dimorphic fungal cell

The present fungal host cells can be used to express any prokaryotic or eukaryotic heterologous peptide or protein of interest, and is preferably used to express  
5 eukaryotic peptides or proteins. Of particular interest for these species is their use in expression of heterologous proteins, especially fungal enzymes.

It will be understood by those skilled in the art that the term "fungal enzymes" includes not only native fungal enzymes, but also those fungal enzymes which have  
10 been modified by amino acid substitutions, deletions, additions, or other modifications which may be made to enhance activity, thermostability, pH tolerance and the like. However, the present host cells may also be used in recombinant production of proteins, which are native to the host cells. Examples of such use include, but are not limited to, placing a Mucor native protein under the control of a different pro-  
15 moter to enhance expression of the protein, to expedite export of a native protein of interest outside the cell by use of a signal sequence, or to increase copy number of a protein which is normally produced by the subject host cells. Thus, the present invention also encompasses, within the scope of the term "heterologous protein", such recombinant production of homologous proteins, to the extent that such  
20 expression involves the use of genetic elements not native to the host cell, or use of native elements which have been manipulated to function in a manner not normally seen in the host cell.

The gene for the desired product functionally linked to promoter and terminator  
25 sequences may be incorporated in a vector containing the selection marker or may be placed on a separate vector or plasmid capable of being integrated into the genome of the host strain. The vector system may be a single vector or plasmid or two or more vectors or plasmids, which together contain the total DNA to be integrated into the genome. Vectors or plasmids may be linear or closed circular  
30 molecules.

According to one preferred embodiment of the present invention, the host is transformed with two vectors, one including the selection marker and the other comprising the remaining heterologous DNA to be introduced, including promoter,  
35 the gene for the desired protein and transcription terminator and polyadenylation



sequences. Also the gene for the control of the dimorphic shift flanked by expression signals identical or different from the above allowing for regulated expression.

The skilled artisan will recognize that the successful transformation of the host species described herein is not limited to the use of the vectors, promoters, and selection markers specifically exemplified. For example, although the *leuA* selection marker is preferred, other useful selection markers include, but are not limited to, the *pyrG*, *met* and *crgA*, as well as markers conferring resistance to carboxin, oligomycin, G-418, zeocyn, and hygromycin B.

When there is provided a dimorphic fungal cell comprising

- i) at least one nucleotide sequence encoding a desirable gene product, and operably linked thereto, and
- ii) at least one further nucleotide sequence comprising a further expression signal capable of directing the expression in a dimorphic fungal cell of the at least one nucleotide sequence encoding the gene product, wherein said further expression signal is regulatable, during growth of the dimorphic fungal cell, by one or more of
  - a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,
  - b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
  - c) the growth phase of the dimorphic fungal cell, and
  - d) the growth rate of the dimorphic fungal cell.

wherein the nucleotide sequence encoding the gene product and the further nucleotide sequence comprising the regulatable expression signal are not natively associated,

5 the dimorphic cell preferably further comprises an isolated polynucleotide comprising

- 10 i) a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- ii) a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

15

wherein the first and second nucleotide sequences are not natively associated.

The dimorphic fungal cell preferably belongs to the class of Zygomycetes, including the order of Mucorales, including a genus selected from the group of  
 20 genera consisting of Mucor, Thermomucor, Rhizomucor, Mycotypha, Rhizopus, and Cokeromyces, preferably the genus Mucor. Within the genus Mucor, the cell is preferably selected from the group of Mucor species consisting of *M. racemosus*; *M. hiemalis*, *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and *M. subtilellissimus*, preferably *M. racemosus*.

25

In one embodiment, the at least one further nucleotide sequence comprising the further expression signal is selected from the group consisting of

- 30 i) a polynucleotide comprising nucleotides 1 to 741 of SEQ ID NO:9,
- ii) a polynucleotide comprising or essentially consisting of the promoter region of *gpd1* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14066; and

- iii) a polynucleotide comprising at least one fragment of SEQ ID NO:9,  
wherein said fragment
- 5 a) is capable of directing gene expression in a dimorphic fungal cell;  
and/or
- b) is regulatable, during growth of the dimorphic fungal cell, by at least  
one factor capable of regulating gene expression directed by SEQ ID  
NO:9; and
- 10 iv) a polynucleotide, the complementary strand of which hybridizes, under  
stringent conditions, with a polynucleotide as defined in any of (i), (ii) and  
(iii), wherein said polynucleotide
- 15 a) is capable of directing gene expression in a dimorphic fungal cell;  
and/or
- b) is regulatable, during growth of the dimorphic fungal cell, by at least  
one factor capable of regulating gene expression directed by SEQ ID  
20 NO:9,

and the complementary strand of such a polynucleotide.

Accordingly, there is provided in one embodiment at least one further nucleotide  
25 sequence comprising nucleotides 1 to 741 of SEQ ID NO:9,

In another embodiment there is provided a polynucleotide comprising or essentially  
consisting of the promoter region of *gpd1* of *Mucor racemosus*, as deposited with  
DSMZ under accession number DSM 14066.

30 In a still further embodiment, there is provided a polynucleotide comprising at least  
one fragment of SEQ ID NO:9, wherein said fragment

- a) is capable of directing gene expression in a dimorphic fungal cell;  
35 and/or

- b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:9.

5

In a still further embodiment, there is provided a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide

10

- a) is capable of directing gene expression in a dimorphic fungal cell; and/or

- b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:9.

15

There is also provided an embodiment, wherein the at least one further nucleotide sequence comprising the further expression signal is selected from the group consisting of

20

- i) a polynucleotide comprising nucleotides 1 to 755 of SEQ ID NO:10,
- ii) a polynucleotide comprising or essentially consisting of the promoter region of *prnC* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14067; and
- iii) a polynucleotide comprising at least one fragment of SEQ ID NO:10, wherein said fragment

25

30

- a) is capable of directing gene expression in a dimorphic fungal cell; and/or

- b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:10; and

35

iv) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide

5

a) is capable of directing gene expression in a dimorphic fungal cell; and/or

10

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:10,

and the complementary strand of such a polynucleotide.

15

Accordingly, there is provided in one embodiment at least one further nucleotide sequence comprising nucleotides 1 to 755 of SEQ ID NO:10.

In another embodiment there is provided a polynucleotide comprising or essentially consisting of the promoter region of *prnC* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14067.

20

In a still further embodiment, there is provided a polynucleotide comprising at least one fragment of SEQ ID NO:10, wherein said fragment

25

a) is capable of directing gene expression in a dimorphic fungal cell; and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:10.

30

In a still further embodiment, there is provided a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide

35

a) is capable of directing gene expression in a dimorphic fungal cell;  
and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least  
one factor capable of regulating gene expression directed by SEQ ID  
NO:10.

5

10

The dimorphic fungal cell is preferably one, wherein the expression in said cell of the nucleotide sequence encoding the gene product results in the production of an increased amount of the gene product as compared to the production of the gene product in an at least substantially identical dimorphic fungal cell under substantially identical conditions, when the nucleotide sequence encoding the gene product is operably linked to the expression signal natively associated therewith.

15

20

25

30

The amount of the gene product produced is preferably increased by a factor of at least 1.02, for example by a factor of at least 1.05, such as a factor of at least 1.10, for example by a factor of at least 1.15, such as a factor of at least 1.20, for example by a factor of at least 1.25, such as a factor of at least 1.30, for example by a factor of at least 1.35, such as a factor of at least 1.40, for example by a factor of at least 1.45, such as a factor of at least 1.50, for example by a factor of at least 1.60, such as a factor of at least 1.70, for example by a factor of at least 1.80, such as a factor of at least 1.90, for example by a factor of at least 2.0, such as a factor of at least 2.5, for example by a factor of at least 3.0, such as a factor of at least 4.0, for example by a factor of at least 5.0, such as a factor of at least 7.5, for example by a factor of at least 10, such as a factor of at least 15, for example by a factor of at least 20, such as a factor of at least 25, for example by a factor of at least 50, such as a factor of at least 100, for example by a factor of at least 150, such as a factor of at least 200, for example by a factor of at least 250, such as a factor of at least 300, for example by a factor of at least 400, such as a factor of at least 500.

35

The at least one nucleotide sequence encoding the gene product and the operably linked to the at least one further nucleotide sequence comprising an expression signal are preferably located on a chromosomal replicon or an extrachromosomal replicon including an expression vector.

The nucleotide sequence encoding a gene product and/or the further nucleotide sequence comprising an expression signal is preferably derived from a fungal cell, including a dimorphic fungal cell, including a fungal cell belonging to the class of Zygomycetes, including a fungal cell belonging to the order of Mucorales, such as a  
5 genus selected from the group of genera consisting of *Mucor*, *Mycotypha*, and *Cokeromyces*, including the *Mucor* species consisting of *M. racemosus*; *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and *M. subtilissimus*.

The gene product is either homologous or heterologous to the dimorphic fungal cell,  
10 and the gene product may be located either in the cytoplasm of the dimorphic fungal cell or, when the nucleotide sequence encoding the gene product is preceded by a signal sequence encoding a signal peptide capable of mediating secretion of the gene product across the extracellular membrane of the dimorphic fungal cell, or located in the growth medium following secretion.

15 To avoid the necessity of disrupting the cell to obtain the expressed product, and to minimize the amount of possible degradation of the expressed product within the cell, it is preferred that the product be secreted outside the cell. To this end, in a preferred embodiment, the gene of interest is linked to a signal peptide, which can  
20 direct the expressed product into the secretory pathway. The signal peptide may be derived from genes for any secreted protein from any organism, or may be the native signal peptide. Among useful available sources for such signal peptide are a *Mucor* glucoamylase or a lipase or proteinase gene from *Mucor racemosus* or *Rhizomucor miehei*. As an alternative, the signal peptide native to the gene being  
25 expressed may also be used.

A signal sequence is a polynucleotide sequence encoding an amino acid sequence which, when operably linked to the amino-terminus of a heterologous polypeptide, permits the secretion of such heterologous polypeptide from the host fungal cell.  
30 Signal sequences may be the signal sequence normally associated with the homologous or heterologous polypeptide (i.e., a native signal sequence), or it may be derived from other sources (i.e., a foreign signal sequence), or it may be synthetic. Signal sequences are operably linked to a heterologous polypeptide either by utilizing a native signal sequence or by joining a DNA sequence encoding a  
35 foreign signal sequence to a DNA sequence encoding the homologous or

heterologous polypeptide in the proper reading frame to permit translation of the signal sequence and heterologous polypeptide. Any signal sequence capable of permitting secretion of a polypeptide in a fungal host cell including a dimorphic fungal cell is contemplated by the present invention.

5

In addition to signal sequences, a DNA encoded precursor peptide may also be present. When positioned in the amino terminal end, such a precursor is known in the art as a propeptide or a prepropeptide. The precursor peptide may also be located in the carboxy terminal end, or in any other location of the mature  
10 homologous or heterologous polypeptide. When a precursor peptide is present, the resultant polypeptide is called a precursor polypeptide. In one embodiment, the precursor polypeptide is a zymogen. Zymogens are biologically inactive proteolytic enzymes and can be converted to mature active polypeptides by catalytic or autocatalytic cleavage of the precursor peptide from the zymogen.

15

A heterologous polypeptide is a polypeptide which is not normally expressed and secreted by the filamentous fungal host cell used to express that particular polypeptide. Heterologous polypeptides include polypeptides derived from prokaryotic sources (e.g., amylases from *Bacillus* species, alkaline proteases from  
20 *Bacillus* species, and various hydrolytic enzymes from e.g. *Pseudomonas*, etc.), polypeptides derived from eukaryotic sources (e.g., bovine chymosin, human tissue plasminogen activator, human growth hormone, human interferon, urokinase, human serum albumin, factor VIII etc.), and polypeptides derived from fungal sources other than the expression host (e.g., glucoamylase from *A. niger* and  
25 *Humicola grisea* expressed in *A. nidulans*, the carboxyl protease from *Mucor miehei* expressed in *A. nidulans*, etc.).

Heterologous polypeptides also include hybrid polypeptides which comprise a combination of partial or complete polypeptide sequences derived from at least two  
30 different polypeptides each of which may be homologous or heterologous with regard to the fungal expression host. Examples of such hybrid polypeptides include: 1) DNA sequences encoding prochymosin fused to DNA sequences encoding the *A. niger* glucoamylase signal and pro sequence alone or in conjunction with various amounts of amino-terminal mature glucoamylase codons, and 2) DNA sequences  
35 encoding fungal glucoamylase or any fungal carboxy protease, human tissue



plasminogen activator or human growth hormone fused to DNA sequences encoding a functional signal sequence alone or in conjunction with various amounts of amino-terminal propeptide condons or mature codons associated with the functional signal.

5

The gene product is preferably selected from the group of gene products consisting of catalase, laccase, phenoloxidase, oxidase, oxidoreductases, cellulase, xylanase, peroxidase, lipase, hydrolase, esterase, cutinase, protease and other proteolytic enzymes, aminopeptidase, carboxypeptidase, phytase, lyase, pectinase and other pectinolytic enzymes, amylase, glucoamylase, alpha-galactosidase, beta-galactosidase, alpha-glucosidase, beta-glucosidase, mannosidase, isomerase, invertase, transferase, ribonuclease, chitinase, mutanase and deoxyribonuclease. Additionally preferred gene products are proteins and enzymes needed for processing of edible or drinkable products, antigens and vaccine components, therapeutic proteins, peptides and hormones.

15

### Examples

The following examples illustrate preferred embodiments of the present invention without limiting the invention to such embodiments. The below table lists the strains deposited with DSMZ.

20

STRAIN	OR-GANISM	GENETIC MARKERS, ETC	RELEVANT FRAGMENT	DSMZ AC-CESSION NO.
pkar13b-1	E. coli	pCR2.1 vector, Ap, Km	Deg PCR pkar (174 bp)	DSM14061
pKAR1	E. coli	YRp17, Ap	Genomic library clone containing the full length <i>pkar</i> gene (~2 kb)	DSM14062
Fus3-4	E. coli	pCR2.1 vector, Ap, Km	Deg. PCR <i>mpk1</i> (541 bp)	DSM14063
Ste12-2b	E. coli	pCR2.1 vector,	Deg PCR	DSM14064

		Ap, Km	<i>ste12</i> (384 bp)	
UPO159	<i>E. coli</i>	pCR2.1 vector, Ap, Km	Deg PCR <i>ste20</i> (~600 bp)	DSM14065
UPO160	<i>E. coli</i>	pCR2.1 vector, Ap, Km	<i>gpd1P</i> (740 bp)	DSM14066
UPO129	<i>E. coli</i>	pCR2.1 vector, Ap, Km	P1 ( <i>prnCP</i> ) (780 bp)	DSM14067
UPO627	<i>M. racemosus</i>	pEUKA4:: <i>gox1</i>	Gpd1P:: <i>gox</i> cassette, <i>trpCtt</i> , <i>leuA</i>	DSM14068
UPO842	<i>M. racemosus</i>	pEUKA8:: <i>gox1</i>	<i>prnCP</i> :: <i>gox</i> cassette, <i>trpCtt</i> , <i>leuA</i>	DSM14069

### Example 1

#### 5 Direct monitoring the dimorphic shift in *Mucor racemosus*

The dimorphic fungal cell *Mucor racemosus* is capable of undergoing a dimorphic shift when responding to environmental cues. The shift involves a change of morphology from e.g. a multinucleated cell having a unicellular, essentially spherical morphology, to e.g. a filamentous fungal cell characterised by an aseptate mycelium comprising multinucleated cells.

Following a shift from anaerobic to aerobic conditions, a transition gradually occurs from a unicellular, essentially spherical morphology to filamentous structures characterised by an aseptate mycelium comprising multinucleated cells occurs.

The present example illustrates the monitoring of this morphogenetic process on single cells.

### Materials and methods

M. racemosus R7B (ATCC 90680), a leucine auxotrophic strain of M. racemosus (syn. circinelloides ATCC1216b) was used. Flask cultures were grown in YPG (complete medium) supplemented with 2 % glucose. Anaerobic growth was achieved by bubbling a mixture of N<sub>2</sub>/CO<sub>2</sub> (30 %:70%) into the medium while stirring. During exponential growth, samples were taken into a 6-well microtiter plate and micrographs were taken on the same field at 10-min time intervals for 10 h using a microscope linked to a CCD camera.

### Results

During anaerobic growth, M. racemosus displays typical spherical and multipolar yeast growth. Upon shift to aerobic conditions a phase of growth in diameter is observed (Fig. 1 and 2, panels 1 to 18), representing the first 3 h after the shift. Subsequently, numerous protruding structures are visible (Fig. 2 and 3, panels 19-32) which evolve into hyphae. Hyphae are structures that show a HGU of more than zero (value for yeast growth). Hyphal development (i.e., elongation) occurs rapidly (Fig. 3, panels 33-40). Branching of hyphae becomes evident and proliferates following the first 7 h after the shift (Fig. 3 and 4, panels 41-60).

### **Example 2**

#### **Molecular Analysis of the Control of Dimorphism in *Mucor racemosus***

The present example illustrates selected mechanisms underlying fungal dimorphism with emphasis in M. racemosus.

### Materials and Methods

*Strains, media and cultivation.* M. racemosus R7B (ATCC 90680), a leucine auxotrophic strain of M. racemosus (syn. circinelloides ATCC1216b) was used as a recipient for transformation experiments and for expression studies. Flask cultures were grown in YNB (minimal medium) or YPG (complete) supplemented with 2 or 5 % glucose. Also, defined SIV medium (Sutter 1975) supplemented with 2 % casamino acids was used. E. coli DH5 $\alpha$  was used for cloning. For the analysis of *pkar* expression, 5 mM dbcAMP (Sigma) was added to aerobic growing cultures during exponential growth.

### Fermentation

Fermentation of *M. racemosus* was carried out using a 2 liter bioreactor and ADI 1035 Bio Console (Applikon bioreactor systems, Applikon, The Netherlands).

5 Cultures were inoculated with  $5 \times 10^5$  spores pr. ml. Strains were grown at 28°C, pH 5.0 and stirred at 200 rpm. Under aerobic growth conditions the culture was sparged with atmospheric air (0.5 vvm), whereas anaerobic conditions was achieved by sparging with 70% N<sub>2</sub>/30% CO<sub>2</sub> (0.5 vvm). Biomass was determined as g cell dry weight pr. kg culture. Glucose in culture supernatant was determined using Glucose (HK) (Sigma Diagnostics).

10

### DNA manipulations

DNA isolation from *M. racemosus* was carried using the Fast DNA kit for soil (BIO101 Inc, CA, USA) using frozen mycelium or yeast cells. All DNA manipulations were carried out according to the manufacturer's recommendations and standard protocols (Sambrook et al. 1989). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase and other molecular biology reagents were from New England Biolabs and Life Technologies. Plasmid pCR2.1 (InVitrogen) was used for the cloning of PCR fragments. PCR was carried out in a GeneAmp PCR Amplifier 2400 (Perkin Elmer), using Taq Polymerase (Life Technologies). DNA sequencing was performed in an ALF Express (Pharmacia) using Cy5 labeled primers as recommended (Table 1).

20

Table 1. Primers used in this example

Primer	Sequence (5' to 3')
pkar-1	GGN GAY TAY TTY TAY GTN GTN GAR
pkar-3	RAA NGT NAC NCK RTC NAR NGC CCA
pkar-pe-1	GGT TGC TCA GCG CAG TAT TCG
pkar-seq1.rev <sup>1</sup>	XGC TGT TGT CCT TAT CTT GGC
pkar-seq2.fwd	XGA CTC TGT GCT TTG GGC TCT GG
pkar-seq3.rev	XCC ACA TCC AGG TAC TGC TCT TCG

pkar-seq4.rev	XGC CAA GAA GAC GAG TGA ATG CC
pkar-seq5.fwd	XTA ACG AAT ACT GCG CTG AGC
pkar-seq6.rev	XCT TGT GCC TGA GAT TTG GGG
pkar-seq7.rev	XCC TCC AAA GTA GGA TCC TCG
pkar5'-EUKA4 <sup>2</sup>	ACT GCC <b>TCG AGA</b> TGA TCA CTG ACG AAC ATC CGT TTG
pkar3'-EUKA4 <sup>2</sup>	ACG CTA <b>GCG GCC GCC</b> GCC TGC GCT TTG AGG TGG AGG CTC ATC
pKAR-ip.fwd	GAC GGG TAT GAT TCA CAG TAT G
pKAR-ip.rev	GTA ACT CTG TCA ACA GCT GCG TG
pkar5'-Sfi1 <sup>2</sup>	AGT CAG CTG <b>GCC ATG CAG GCC</b> ATG ATC ACT GAC GAA CAT CCG TTT G
pkar3'-Sfi1 <sup>2</sup>	AGT CAG CTG <b>GCC ACG TAG GCC</b> TGC GCT TTG AGG TGG AGG CTC ATC
Ste12 5'	AARTTYTTY TNGCNACNGC NCCNGTNAAY TGG
Ste12 3'	RAACCARAAR AANACYTTYT GYTTYTTYTG NGT
FUS3-3'	CAT DAT YTC NGG NGC NCK RTA CCA
FUS3-5'	TAY ATH GTN CAR GAR ATH ATG

<sup>1</sup>X depicts Cy5 labeling (sequencing primers)

<sup>2</sup>*Xho*I (CTCGAG), *Not*I (GCGGCCGC) and *Sfi*I (GGCCATGCAGGCC) sites are shown in bold.

5

Inverse PCR was carried out using primer pKAR-ip.fwd and pKAR-ip.rev (position 2185-2206 and 614-592 in the *pkar* sequence, Table 1) and *Hind*III digested and religated genomic DNA from strain R7B. Subsequent cloning and sequencing of the amplified fragment provided with approx. 500 bp of promoter sequence. Probe labeling was performed using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech). Unincorporated nucleotides were removed using a NICK column (Pharmacia).

10

*Plasmid construction.* The full-length coding region of the *M. racemosus* *pkar* gene spanning from the ATG start codon (position 542-544 in the obtained sequence) to the region downstream of the polyadenylation signal (position 1959-1964) was amplified using primers *pkar*-5'-EUKA4 and *pkar*-3'-EUKA4 (spanning from position 542-566 and 2220-2248, respectively; Table 1). The expected 1.7-kb fragment was cloned into pCR2.1, and transformed into *E. coli*, resulting in plasmid pCR1-*pkar*. DNA of pCR1-*pkar* was digested with *Not*I, purified from an agarose gel and partially digested with *Xho*I. The 1.7-kb full-length *pkar* fragment was purified from an agarose gel and cloned into *Xho*I-*Not*I digested and phosphatase-treated pEUKA4. In the resulting plasmid, named pEUKA4::*pkar* (Fig. 8A), expression of *pkar* is under control of the *M. racemosus* *gpd1P* promoter, which is induced in response to the presence of glucose or galactose (Wolff et al. 2001).

#### Transformation of *M. racemosus*

Protoplasts formation and transformation was performed according to (van Heeswick and Roncero 1984) with the following modifications. Protoplasts were prepared by enzymatic treatment of germlings with a mixture of 125 µg chitosanase-RD (US Biological) and 5 Units chitinase (Sigma) in a final volume of 2 ml. Cell wall digestion was carried out for 2-3 h at 28 °C. Typically, 1-10 µg DNA were used per transformation. Individual transformants were transferred to YNB plates after 5-7 days.

#### RNA isolation and analysis

RNA isolation was carried out using a FastRNA kit (BIO101) and extraction with acid Phenol:chloroform (Sigma). Total RNA was loaded onto formaldehyde containing agarose gel, subjected to electrophoresis and transferred to GeneScreen membrane (Du Pont) as described (Arnau *et al.*, 1996). Northern blots were used to measure induction of expression using a Cyclone Storage Phosphor System (Packard) and the OptiQuant image analysis software. A linear dynamic range of 5 orders of magnitude with only a 5% standard deviation is possible with the above apparatus. Dilution series of the RNA preparations (typically 20, 10 and 5 µg total RNA per sample) were used to estimate induction levels. Primer extension was carried out using radioactively end-labeled primer *pkar*-pe-1 (Table 1), complementary to position 98-78 downstream of the *pkar* ATG start codon. A sequence ladder was obtained using the same primer and pEUKA4::*pkar* DNA as template using the

Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB-Amersham).

#### Genomic library screening

5 The *M. racemosus* genomic library (van Heeswick and Roncero 1984) was used to screen for clones that contained *pkar* sequences using the *pkar13b-1* fragment as a probe (Fig. 6A). Colony hybridization was carried out as described (Sambrook et al. 1989).

#### Sequence analysis and accession numbers

10 The DNASTAR package (Lasergene) was used for multi-alignments. The EMBL outstation Fasta3 and the NCBI Blast search programs were used for DNA and protein homology analysis. The sequence of the *M. racemosus pkar*, *ste20*, *mpk1* and *ste12* genes described here have been deposited in the databases with accession numbers AJ400723, AJ309732, AJ309731 and AJ400724, respectively.

### 15 **Results and Discussion**

#### Cloning of the *M. racemosus pkar* gene

Several fungal genes encoding the regulatory subunit of the protein kinase A (PKAR) are present in the databases. The high level of sequence homology allowed the design of degenerate primers (Table 1) derived from the GDFFYVVE and  
20 WALDRNTS regions (positions 219-226 and 272-279 in the *M. racemosus* PKAR protein, see below) and the PCR amplification of a 183-bp fragment, named *pkar13b-1*. Sequence analysis and database searches identified *pkar13b-1* as highly homologous to known fungal and eukaryotic *pkar* genes. Using *pkar13b-1* as a probe, a positive clone, pKAR1 was identified from a *M. racemosus* genomic  
25 library (van Heeswijck and Roncero 1984). Sequence analysis of the 2-kb insert in pKAR1 showed that it contained a chromosomal insert encompassing the full-length *M. racemosus pkar* gene including 40 bp upstream of the ATG start codon (Fig. 6). Further characterization using inverse PCR allowed the characterization of the upstream region of *pkar* including 541 bp of the promoter region (Fig. 6).

30

The promoter region includes two canonical CAAT boxes (positions 377-380 and 459-462, respectively), a TATA box (position 492-496 in the sequence) and a CT rich stretch (position 517-535) adjacent to the ATG start codon (position 542-544). Interestingly, multiple putative GATA boxes (positions 127-130, 178-181, 191-194

and 398–401, respectively) are also present in the promoter region suggesting that *pkar* expression in *M. racemosus* might be regulated through GATA factors (Wilson and Arst, 1998).

- 5 The *M. racemosus pkar* gene includes two introns, one at the 5'-end of the coding region and the other separating the coding region corresponding to the two cAMP-binding domains (see below). The presence of introns in this latter region is unusual and no introns have been reported in the related *M. rouxii pkar* gene although other fungal homologues include several introns (Bruno et al., 1996; Sorol et al., 2000).
- 10 The *pkar* gene encodes a putative 427 aa protein with an estimated molecular weight of 48.7 kDa. These data correlate with the estimated size (51 kDa) for the *M. racemosus* PKAR using binding to radioactively labeled cAMP (Orlowski 1991). The *M. racemosus* PKAR is 45 % and 36 % identical to the PKAR of *Blastocladiella emersonii* and *Saccharomyces cerevisiae*, respectively (Fig. 7). Strikingly, only 66 %
- 15 identity was found in the cAMP binding domains of the two *Mucor* PKAR proteins (Sorol et al., 2000; Fig. 7). It is tempting to suggest that the observed differences in the requirements for yeast and filamentous growth in *M. racemosus* and *M. rouxii* are due to the anomalously high sequence divergence in PKAR resulting in a different *in vivo* specificity.
- 20 Some of the conserved features of known eukaryotic PKARs are also present in the *M. racemosus* homologue. The PKAR dimerization region is not highly conserved between various PKARs. The kinase inhibitor domain is a highly conserved six-residue sequence (RRtSVs) that acts as an inhibitor of PKAC kinase activity and
- 25 mediates interaction between PKAR and PKAC subunits. The well-conserved kinase inhibitor domain is present in the *M. racemosus* PKAR (RRTSVK at position 144–149 in the aa sequence, Fig. 7). The partial sequence available from the PKAR of the related fungal cell *M. rouxii* does not include this domain (Sorol et al., 2000) and therefore comparison of the PKAR kinase inhibitor domain between these two fungi
- 30 awaits. Two near-duplicate cAMP-binding domains are also found in typical PKARs and both domains are present in the *M. racemosus* PKAR (sFGELALmynAPRAATii and yFGELALIndAPRAATvv, at positions 247–264 and 369–386, respectively, in the aa sequence, Fig. 7).



### Analysis of *pkar* expression in *M. racemosus* during the dimorphic shift

Contrary to the situation in other dimorphic fungi like *C. albicans*, high levels of cAMP are associated in *M. racemosus* with yeast growth and a rapid decrease in cAMP levels accompanies the transition from yeast to filamentous growth (Orlowski  
 5 1991). These observations suggested that PKAC might be released and active during yeast growth, while PKAR repression might function during filamentous growth. If this was the case, expression of *pkar* should be induced during the dimorphic shift from yeast to filamentous growth. Northern blot analysis using a *pkar*  
 10 specific probe confirmed that the level of *pkar* expression is low during anaerobic yeast growth and is induced after the shift to aerobic filamentous growth (Fig. 6B). Quantification of mRNA levels indicated that a 2-fold induction of expression occurs during aerobic growth. A single 1.4-kb transcript was observed in all growth experiments, correlating with the deduced size of the coding region. This is in  
 15 contrast to the *Neurospora crassa* *pkar* homologue, the *mcb* gene (Bruno et al. 1996). In this case, two transcripts were observed and a 500-bp long non-translated upstream region of the large transcript included two introns.

In *S. cerevisiae*, cAMP-dependent signal transduction is induced by glucose. To investigate if a similar mechanism was present in *M. racemosus*, the expression of  
 20 *pkar* was analyzed in anaerobic cultures shifted to aerobic growth with the simultaneous addition of different glucose concentrations. No differences in expression levels were observed in the conditions used (0-10 % (w/v) glucose), indicating that glucose does not alter *pkar* expression levels (Fig. 6B). Thus, a higher glucose concentration does not lead *per se* to an increase in PKA levels via an alteration in  
 25 the level of cAMP.

In a complementary study, the expression of *pkar* was followed during aerobic (filamentous) growth before and after the addition of dbcAMP. This cAMP analogue is membrane permeable and its addition results in PKA activation. As shown in  
 30 Table 2, *pkar* expression is rapidly and gradually induced following dbcAMP addition.

**Table 2.** Induction of *pkar* expression during aerobic growth in response to dbcAMP

Sample	Total Counts <sup>2</sup>	Fold induction <sup>3</sup>
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T:0 <sup>1</sup>	419	1.0
T:15 min	951	2.3
T:30 min	1728	4.1
T:60 min	2235	5.3
T:120 min	2392	5.7
T:180 min	2966	7.1

<sup>1</sup>T:0: before the addition of 5 mM dbcAMP; T:15 min: 15 min after the addition, etc.

<sup>2</sup>Background counts were subtracted

<sup>3</sup>Values normalized with respect to counts at T:0 (1.0)

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After three hours, a sevenfold induction was observed compared to the level observed before dbcAMP addition. Since aerobic growth is associated with a low level of PKA (or cAMP) in *M. racemosus*, the dramatic change in cAMP levels results in an immediate increase in *pkar* expression. Under these conditions, the enhanced level of PKAR may counteract the excess cAMP during the period of transition from aerobic filamentous to yeast growth that follows after addition of dbcAMP to aerobic cultures (Orlowski 1991).

15 In order to investigate the role of PKAR during filamentous growth in *M. racemosus*, a strain (KFA121) was constructed that contains the *pkar* gene under the control of the *gpd1* promoter (*gpd1P*), recently isolated and characterized in our group (Wolff et al., 2001). The *gpd1P* is induced by glucose or galactose and a correlation between the concentration of sugar in the medium and the level of *gpd1P*-driven expression in *M. racemosus* has been observed (Wolff et al., 2001). Therefore, strain KFA121 was grown in liquid YNB medium containing 5 % glucose and the expression of *pkar* was compared to a vector control strain by Northern blot. As shown, a high level of expression is observed in KFA121 (Fig. 8B, lane 2). Furthermore, extensive *pkar*-mRNA degradation was observed in KFA121 suggesting that a regulatory mechanism might be triggered in *M. racemosus* KFA121 to prevent excess mRNA. In fact, specific RNA degradation has been reported in this fungal cell during heterologous expression of the *E. coli* GUS reporter (Garcia-Castillo et al., 2000). However, degradation of *pkar*-mRNA was not detected in the vector control strain grown under the same conditions (Fig.8, lane 1), thus precluding non-specific degradation. Primer extension experiments demonstrated that transcription

of the plasmid-borne *pkar* gene starts at the original transcription start site of the *M. racemosus gpd1* gene (Fig. 8B; Wolff et al., 2001).

5 No morphological differences were observed in aerobic liquid medium between KFA121 and the control strain. However during growth on plates, KFA121 displayed a small colony phenotype. Microscopic examination of these colonies showed that branching at the hyphal tips was more frequent in KFA121 (Fig. 8C). Taken together, these results indicate that PKAR plays a role in filamentation and branching in *M. racemosus* during aerobic growth. Recently, it has been postulated that the  
10 PKA tetrameric complex (2 x [PKAR-PKAC]) might also display some regulatory role in *M. rouxii* where PKAR would participate in the specificity of the phosphorylation event (Zaremborg et al., 2000). The observed colony morphology in KFA121 suggests a role of PKAR in filamentation.

15 During anaerobic growth, the presence of non-germinated spores was observed both in solid and liquid medium (data not shown). Whether this is due to a titration of free PKAC subunits thereby hindering normal yeast growth remains to be examined.

Cloning of a fragment of the *M. racemosus ste12* gene

20 STE12 is a transcription factor that participates in the MAPK-dependent signal transduction pathway in *S. cerevisiae*, *C. albicans* and *C. neoformans* leading to filamentation (Fig. 5; Liu et al., 1994; Yue et al., 1999). STE12 acts downstream in the signal pathway hierarchy and a null mutant may only be affected in a limited number of functions, as opposed to e.g., a *pkar* mutant. In *C. albicans*, a *ste12* null  
25 mutant strain is defective in filamentation (Liu et al., 1994). The STE12 polypeptide consists of an N-terminal domain involved in DNA recognition and binding and a C-terminal domain involved in protein-protein interaction (Yue et al., 1999). Sequence homology is restricted to the DNA-binding domain. To investigate whether *M. racemosus* possesses a *ste12* homologue, degenerate PCR was carried out using  
30 primers designed from the most conserved sequence of the N-terminal region of available *ste12* genes (KFFLATA and QKKQKVF positions 44-50 and 151-157 in the *S. cerevisiae* STE12 protein sequence, Fig. 9). An expected 384-bp fragment, *ste12b-1*, was obtained using R7B DNA as template, indicating the lack of introns in the cloned region. Cloning into pCR2.1 and sequencing confirmed that an uninterrupted coding region was present in *ste12b-1*. Database searches using *ste12b-1*  
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showed the highest degree of homology to the N-terminal region of the *C. albicans* CPH1, the STE12 homologue (Fig. 9), confirming that the cloned fragment is part of the *M. racemosus ste12* gene. Several unsuccessful attempts were performed to screen the genomic library using this fragment as a probe in order to clone the full-length gene.

#### Cloning of a fragment of *M. racemosus mpk1* (mitogen-activated protein kinase 1)

In *S. cerevisiae* and *C. albicans*, STE12 is activated by the MAP kinases KSS1 or CEK1, respectively, triggering filamentous growth (Fig. 5). A homologous function should exist in *M. racemosus* and might be involved in the regulation of STE12 thereby participating in filamentation. Two highly conserved regions present in the majority of cloned MAP kinases (YI/LVQEIMA and YRAPEIM; Lim et al., 1999; Fig. 10) were chosen for primer design. A 541-bp fragment (*fus3-4*) was amplified using R7B DNA as template, whereas a 292 bp fragment was expected from the protein sequence. Sequence analysis showed that *fus3-4* included a fragment of a *M. racemosus* MAP kinase homologue and that three putative introns were present in the sequence obtained (Fig. 10A). The deduced protein sequence showed high identity (70 %) to the *Schizosaccharomyces pombe* SPM1 among other fungal MAP kinases (Fig. 10B).

#### Identification of *M. racemosus ste20* encoding a MAP kinase kinase kinase

In *S. cerevisiae*, STE20 participates in the MAP kinase signaling pathways involved in mating and pseudofilamentation. During the search for gene homologues to fungal hexokinases using degenerated primers, a clone (UPO896) was sequenced and the homology search identified it as a likely *M. racemosus ste20* counterpart. The fragment cloned (634 bp) included sequence derived from one of the primers used at either end (data not shown) and spans two incomplete exons and an intron and the deduced protein sequence shows high homology to known STE20 and related serine/threonine kinases (Fig. 11).

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### Example 3

#### 10 Use of *M. racemosus gpd1* for recombinant protein production

The present example discloses three genes (*gpd1*, *gpd2* and *gpd3*) encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) and their isolation from the dimorphic zygomycete *Mucor racemosus* by PCR using degenerated primers.

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Only transcription of *gpd1* could be detected during vegetative growth under both aerobic and anaerobic conditions, indicating that *gpd1* is the main GPD-encoding gene. The transcription of *gpd1* was significantly higher on fermentable carbon sources than on non-fermentable carbon sources during growth under aerobic conditions, indicating that *gpd1* expression is subjected to carbon catabolite regulation.

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A direct correlation between the abundance of *gpd1* mRNA and the concentration of sugar in the medium was found during anaerobic growth. The *gpd1* promoter was successfully used for recombinant expression of genes of both homologous (*crgA*) and heterologous (*gox1* from *A. niger*) nature. Growth of a *gox1* transformant strain resulted in the secretion of enzymatically active glucose oxidase.

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#### Introduction

Genetic markers and a transformation system based on complementation of leucine auxotrophy have been established in *M. racemosus* (Roncero *et al.*, 1989). However, the availability of other genetic tools such as promoter sequences and alternative selection markers to allow for genetic studies and provide tools for recombinant protein production is very limited. In fact, only a few reports are published where *Mucor* has been used for heterologous protein production (Dickinson *et al.*, 1987; Strøman *et al.*, 1990).

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Glyceraldehyde-3-phosphate dehydrogenase (GPD) is one of the key enzymes in the glycolytic pathway and in many eukaryotic microorganisms the GPD-encoding genes are expressed constitutively and at high levels (Holland and Holland, 1978; Edens, 1984; Waterham *et al.*, 1997; Hirano *et al.*, 1999). The promoter sequences of native GPD-encoding genes have proven useful for efficient expression of heterologous genes in several yeasts and fungi (Bitter and Egans, 1984; Waterham *et al.*, 1997; Van den Hondel and Punt, 1991; Schuren and Wessels, 1994; Van de Rhee *et al.*, 1996; Hirano *et al.*, 2000). With the aim of obtaining a strong homologous promoter allowing efficient recombinant expression in *M. racemosus*, GPD genes were cloned from *M. racemosus*. The characterization of three individual genes (*gpd1*, *gpd2* and *gpd3*) together with the use of the promoter region of the *gpd1* gene for recombinant protein production is presented here.

## Materials and Methods

### Strains and media

The *Mucor racemosus* syn. *circinelloides* strain R7B (ATCC 90680; a leucine auxotrophic derivative of ATCC 1216b) was used throughout this study. *Mucor* was grown in either YPG (Bartnicki-Garcia and Nickerson, 1962), YNB (Lasker and Borgia, 1980), Vogel's medium supplemented with 1.5 g glutamic acid pr. litre (Hoekstra *et al.*, 1996) or SIV medium with 1.5 g glutamic acid pr. litre in place of L-asparagine and supplemented with 5 g casamino acids (DIFCO) pr. litre (Eslava and Alvarez, 1996). All media were supplemented with 1 mg/L niacin amide and 1 mg/L thiamine chloride. In some experiments glucose was replaced by galactose (20 g/L), glycerol (20 ml/L) or ethanol (5 ml/L). The *E. coli* strain Top10 (Invitrogen Corp.) was used in DNA manipulations and grown in LB medium (Sambrook *et al.*, 1989).

### Fermentation

Fermentation of *M. racemosus* was carried out using a 2 liter bioreactor and ADI 1035 Bio Console (Applikon bioreactor systems, Applikon, The Netherlands). Cultures were inoculated with  $5 \times 10^5$  spores pr. ml. Strains were grown at 28°C, pH 5.0 and stirred at 200 rpm. Under aerobic growth conditions the culture was sparged with atmospheric air (0.5 vvm), whereas anaerobic conditions was achieved by sparging with 70% N<sub>2</sub>/30% CO<sub>2</sub> (0.5 vvm). Biomass was determined as g cell dry

weight pr. kg culture. Glucose in culture supernatant was determined using Glucose (HK) (Sigma Diagnostics).

#### PCR with degenerate primers and inverse PCR (IPCR)

5 GPD-encoding genes were cloned using *M. racemosus* R7B chromosomal DNA as template and degenerate primers designed from the well-conserved amino acid sequences: INGFGGRIG and WYDNEYGY. PCR products of 1.1 kb were cloned in pCR2.1 (Invitrogen Corp.) and sequenced, and from the obtained DNA sequence primers for inverse PCR (IPCR) were designed. IPCR was performed as described  
10 (*Ochman et al.*, 1990).

#### Colony hybridization

A *Mucor racemosus* genomic library constructed in the yeast-*E. coli* shuttle vector YRp17 was obtained from Professor M. I. G. Roncero (Heeswijk and Roncero,  
15 1984). *E. coli* clones were screened as described (*Sambrook et al.*, 1989) using a *gpd2*-specific oligonucleotide as probe.

#### DNA sequencing

Sequence reactions were carried out using cycle sequencing with fluorescent  
20 primers on an ALF Express sequencer (Pharmacia) according to the manufacturer's instruction. The obtained DNA sequences were deposited at EMBL (Accession nos. AJ293012, AJ293013 and AJ293014). The deduced amino acid sequences encoded by *M. racemosus gpd1*, *gpd2* and *gpd3* were compared with those of GPD proteins from other fungi using the Clustal alignment feature of the MegAlign program  
25 (DNASTAR, Lasergene Inc., Madison, WI).

#### Construction of expression vector

Vectors based on pLeu4 (Arnau and Strøman, 1993) were constructed using standard techniques. The construction of pEUKA4 containing Ap<sup>R</sup> and *leuA* as  
30 selection markers for selection in *E. coli* and *M. racemosus*, respectively, the *gpd1* promoter (nucleotides -741 → -1 in Fig. 12) and the *A. nidulans trpC* terminator (EMBL Accession no. X02390, nucleotides 3563-4167) will be described elsewhere. The *crgA* gene (EMBL Accession no. AJ25099, nucleotides 626-2233) was amplified from *M. racemosus* chromosomal DNA using PCR and the resulting fragment

was cloned into the *Xho*I and *Not*I sites of pEUKA4 giving rise to pEUKA4-crgA. Similarly, a PCR generated fragment containing *gox1* from *A. niger* (EMBL Accession no. X16061, nucleotides 40-1857) was cloned into pEUKA4 giving rise to pEUKA4-*gox1*. The complete nucleotide sequences of pEUKA4-crgA and pEUKA4-*gox1* were deposited at EMBL (Accession nos. AJ305344 and AJ305345, respectively).

#### Primer extension

Primer extension was performed using Primer Extension System (Promega) according to the manufacturer's instruction. The gene specific primers *gpd1*primerREV (CATCCTTGTTGGACTCAGTAGC), *gpd2*primerREV (CTTCAGGGTTAGAGAGAGAAGC) and *gpd3*primerREV (CCTTGGGGTTTTTCGAGGGAGG) were used for primer extension. To determine the transcription start point a sequence reaction was performed using the same primers and run on the same gel as the primer extension products.

#### Northern blot analysis

Total RNA for Northern blot analysis was isolated using FastRNA and FastPrep FP120 (BIO101 Inc., CA, USA). Approximately 10 µg RNA was loaded onto formaldehyde containing agarose gel, subjected to electrophoresis and transferred to GeneScreen membrane (Du Pont) as described (Arnau *et al.*, 1996) with the following modifications when oligonucleotide probes were used: i) Prehybridization and hybridization was performed at 50°C and the buffer was supplemented with 1 M EDTA; ii) The blots were washed 1, 2 and 3 min at room temperature in 6 x SSC, 1% SDS and 1.5 min at 50°C in 1 x SSC, 1% SDS. Probe labelling was performed using Ready-To-Go DNA Labelling Beads (Amersham Pharmacia biotech). Unincorporated nucleotides were removed using a NICK column (Pharmacia).

#### Zymogram

Proteins in culture supernatants or cell extracts were subjected to SDS-PAGE in 14% polyacrylamide gels under native conditions using precast gels (Novex, CA, USA). Cells were lysed using FastProtein Red and FastPrep FP120 (BIO101, Inc., CA, USA). After electrophoresis gels were incubated in the dark with assay reagent (0.1 M glucose, 0.1 mg/ml N-methyl-dibenzopyrazin methylsulphate (Sigma), 0.2

mg/ml 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromid (Sigma) in 100 mM citrate-phosphate buffer pH 6.5) (Sock and Rohringer, 1988). Commercial GOX (Sigma) was used as positive control.

#### Transformation of *M. racemosus*

- 5 Transformation of *Mucor* was performed basically as described (Van Heeswick *et al.*, 1988). Protoplast formation was performed by digestion with 62.5 µg/ml chitosanase RD (US Biologicals, USA) and 12.5 units/ml chitinase (from *Streptomyces griseus*, Sigma).

### 10 **Results and Discussion**

#### Isolation of *gpd* genes

- Potential GPD-encoding genes were isolated from *Mucor racemosus* by PCR using degenerate primers designed from highly conserved regions of GPD sequences from other fungi. The resulting PCR products of approximately 1.1 kb were cloned and sequencing of several independent clones revealed three different sequences (*gpd1*, *gpd2* and *gpd3*) with significant homology to known GPD-encoding genes. The flanking DNA regions were obtained by inverse PCR (*gpd1* and *gpd3*) or by colony hybridization (*gpd2*).

#### Introns

- 20 The genomic regions of *M. racemosus gpd1*, *gpd2* and *gpd3* span 1127, 1213 and 1213 nucleotides, respectively, and contains two, three and two introns, respectively, with an average size of 63 nucleotides (Fig. 12-14). All introns have 5' splice site (GTA) and 3' splice site (PyAG) in agreement with the consensus sequences for introns of filamentous fungi (Ballance, 1990). Further, the two introns in *gpd1* and *gpd3* and one of the three introns in *gpd2* contain sequences similar to the "lariat formation" consensus sequence for filamentous fungi (5' - (G/A)CTAAC-3') (Ballance, 1990). The introns in *gpd1*, *gpd2* and *gpd3* are placed at four different positions, i.e. three of the intron positions are conserved among two genes (Fig. 15A). It has previously been pointed out that the positions of introns are strongly conserved within the group of ascomycetes and basidiomycetes, respectively, but only the position of a single intron is conserved between the two classes (Harmsen *et al.*, 1992). No introns have been found in GPD-encoding genes from ascomycetous yeasts. In contrast, the *gpd* genes from the basidiomycetous yeasts *Phaffia*

rhodomyces and *Cryptococcus neoformans* contain 6 and 11 introns, respectively. A few of these introns are located at positions conserved among the filamentous basidiomycetes, but most of them are placed at unique positions, which may reflect the evolutionary divergence of the yeasts from the filamentous fungi. In *M. racemosus* *gpd1*, *gpd2* and *gpd3*, two of the four intron positions are also found among the basidiomycetes, whereas the other two positions are unique to the zygomycete which suggests closer phylogenetic relation of *Mucor* to basidiomycetes than to ascomycetes.

#### The 5' and 3' flanking regions

The sequences upstream of the coding regions of *gpd1* and *gpd2* contain consensus promoter elements within the expected distances relative to the ATG initiation codon (Fig. 12-13). A putative TATA box (TATAAA) was observed 84 bp and 80 bp upstream of the initiating ATG of *gpd1* and *gpd2*, respectively. Further, CAAT boxes were found 77 and 164 bp upstream of the start codon of *gpd1* and 139 bp upstream of the start codon of *gpd2*. In contrast, these "core promoter" elements in the sequence upstream of *gpd3* were found further upstream of the initiating ATG: a putative TATA box and a putative CAAT box were found 341 and 289 bp upstream of ATG, respectively (Fig. 14). The site of transcriptional initiation of *gpd1* was determined by primer extension to be 34 nucleotides upstream of the translation initiation codon. Primer extension using *gpd2* and *gpd3* specific primers did not result in any products neither with RNA isolated from an anaerobically growing yeast culture nor with RNA isolated from an aerobically growing filamentous culture, indicating that the *gpd2* and *gpd3* genes are not transcribed under these conditions. A pyrimidine region composed of stretches of thymine nucleotides interrupted by cytosine residues was observed immediately upstream of the transcription initiation site of *gpd1* (Fig. 12) as has also been observed for other fungal genes (Gurr, 1988). Similar pyrimidine regions were also observed in the *gpd2* and *gpd3* sequences suggesting putative transcription initiation approximately 30 and 270 nucleotides upstream of ATG, respectively. Sequences conserved between the promoters of *A. nidulans* and *A. niger* GPD-encoding genes, such as *gpd* box, *pgk* box, *qut* box and *qa* box (Punt *et al.*, 1990), are not present in the promoter regions of *gpd1*, *gpd2* and *gpd3*. Consensus polyadenylation sites (AATAAA) were found 51 and 58 nucleotide after the stop codon of *gpd1* and *gpd2*, respectively, whereas the same sequence was found 333 nucleotides downstream of the stop codon of *gpd3*. Overall, the three genes display nucleotide sequences required for transcription and

subsequent processing within the 5' and 3' flanking regions. However, for *gpd3* these sequence elements are found further away from the coding sequence than usually observed for fungal genes, which may result in sub-optimal transcription of *gpd3* or even a non-functional gene.

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#### Amino acid sequence and codon usage

*gpd1*, *gpd2* and *gpd3* are predicted to encode polypeptides of 337, 338 and 339 amino acids, respectively. The three sequences show extensive homology to each other (80-86% identity, Fig. 13) and to GPDs from other species (63-79% identity to known yeast and fungal GPDs). Previous phylogenetic analyses of yeast and fungal GPD sequences have shown clustering of GPDs from ascomycetous yeasts, filamentous ascomycetes and basidiomycetes, respectively, into distinct groups (Verdoes *et al.*, 1997; Harmsen *et al.*, 1992). As an exception the GPD encoded by *gpd1* of the basidiomycete *A. bisporus* falls outside this grouping. The three *M. racemosus* GPD sequences show highest homology to the sequences obtained from the group of basidiomycetes (>70% identity to the GPDs belonging to this group) and to a subset of the sequences obtained from the ascomycetous yeast (>70% identity to GPDs from *S. pombe*, *P. pastoris* and *C. albicans*). In contrast, the homology to the GPD sequences obtained from the group of ascomycetes is lower: for most of the 15 GPD sequences in this group the identity is lower than 70%. A phylogenetic Clustal analysis showed grouping of the *M. racemosus* sequences among the ascomycetous yeast sequences (Fig 14). Thus, amino acid sequence analysis of GPDs indicate a closer phylogenetic relation of *Mucor* to the basidiomycetes and a subset of the ascomycetous yeasts than to the filamentous ascomycetes. The codon usage in *gpd1* and *gpd2* is highly biased with 83% and 81% pyrimidines at the third position, respectively, and 21 unused codons in both genes. Pyrimidines dominate the third nucleotide position, but in contrast to many other fungal genes, U is preferred to C (Gurr *et al.*, 1988). Where a purine is found in the third position, G is used in preference to A. The degree of codon bias in *gpd3* is much lower with 67% pyrimidine at the third position and only 6 unused codons, suggesting that *gpd3* is not a highly expressed gene.

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### Expression of *gpd*

The transcription of *gpd1*, *gpd2* and *gpd3* was analyzed under different growth conditions by Northern blotting using gene-specific oligonucleotides as probes derived from a divergent region (Fig 12-14). *M. racemosus* R7B was grown in fermentor in Vogel's medium with either 2% or 5% glucose. Total RNA was isolated from a culture, growing exponentially as yeasts in the presence of glucose under anaerobic conditions, and from a culture, which had been shifted from anaerobic to aerobic conditions at the time of glucose depletion and further incubated for four hours allowing the initiation of filamentous growth. A high level of expression of *gpd1* was observed in the yeast culture whereas low expression of *gpd1* could be detected in the filamentous culture (Fig. 16). No expression of *gpd2* or *gpd3* was detected in any of the cultures. These data show that *gpd1* is highly expressed under anaerobic growth conditions in presence of glucose and that the expression of *gpd1* is highly regulated in response to either carbon source or anaerobiosis. Further, the data strongly indicate that although all three genes may potentially encode functional GPD, *gpd1* is the major if not the only GPD-encoding gene in *M. racemosus* as *gpd2* and *gpd3* are inactive at least during vegetative growth. In the cultivated/common mushroom *Agaricus bisporus* two genes have been identified, but only one of the two tandemly linked GPD genes is transcriptionally active (Harmsen *et al.*, 1992). Conversely, in the budding yeast *S. cerevisiae* three separate GPD-encoding genes are differentially expressed, indicating that the different isoforms may have distinct cellular roles (Bouchérié *et al.*, 1995). Whether the two genes in *M. racemosus*, *gpd2* and *gpd3* represent functional genes which are only transcribed at specific growth stages (e.g. sporulation or germination), or non-functional pseudogenes remains to be shown.

The transcription of *gpd1* was analyzed under various growth conditions by Northern blotting. *M. racemosus* was grown aerobically in rich medium with different carbon sources in shake flasks. In the presence of glucose, a strong expression of *gpd1* was observed while a significantly lower expression of *gpd1* was observed when glycerol was present in the medium (Fig. 17A). Low expression of *gpd1* was detected when ethanol was added to the medium. These results show that *gpd1* expression is primarily regulated in response to carbon source rather than in response to aerobiosis. In order to analyze further the regulation of *gpd1* expression in response to carbon source, *M. racemosus* was grown as yeast under anaerobic

conditions in rich medium with either glucose or galactose in fermentors. A strong expression of *gpd1* was observed in the presence of high concentrations of glucose or galactose during growth in fermentor (Fig. 17B). The level of mRNA gradually decreased as the sugar was consumed and a low level of transcript was observed after depletion of sugar. However, subsequent addition of glucose to the culture resulted in a rapid increase in *gpd1* expression (Fig 17B, glucose concentration 1.4). These observations indicate that *gpd1* expression in *M. racemosus* is strongly regulated in response to sugar concentration. A slight ( $\leq$ two-fold) decrease in mRNA level was observed during growth in fermentor under anaerobic conditions and after shift to aerobic conditions before glucose was depleted (data not shown). Thus, the transcription of *gpd1* correlated with the concentration of glucose and galactose, whereas anaerobic versus aerobic growth conditions only had a minor effect on transcription. In many other filamentous fungi and yeasts e.g. *A. nidulans*, *A. niger*, *N. crassa*, *K. lactis*, *P. pastoris* and *C. albicans* expression of GPD-encoding genes has been found to be high and only subjected to minor changes during vegetative growth. Therefore, it was somewhat unexpected to find that the expression of the *M. racemosus gpd1* was highly regulated.

#### Recombinant expression

It has previously been shown that recombinant expression of the *crgA* gene, encoding a regulator of carotene biosynthesis, can abolish the light requirement for carotene production in *M. racemosus* (Navarro *et al.*, 2000). In order to test whether the *gpd1* promoter could mediate expression of the homologous gene *crgA*, the 740-bp *gpd1* promoter region was placed upstream of the *crgA* gene in a *M. racemosus* vector. The resulting plasmid (pEUKA4-*crgA*, Fig. 18A) was introduced into *M. racemosus* strain R7B and transformants were incubated in the dark in order to prevent expression from the endogenous light-inducible *crgA*. After 3 days, *crgA* transformants showed a yellow-orange color, in contrast to a control strain, providing evidence that the *gpd1* promoter indeed is able to drive expression of the *crgA* gene resulting in light-independent carotene synthesis (Fig 18B).

In order to study heterologous gene expression in *M. racemosus*, the gene encoding glucose oxidase (GOX) from *Aspergillus niger* (*gox1*, Frederick *et al.*, 1990) was placed under the control of the *gpd1* promoter in plasmid pEUKA4-*gox1* (Fig. 18A).

*M. racemosus* R7B transformed with pEUKA4-*gox1* was analyzed for GOX produc-



tion in shake flask experiments. Analysis of culture supernatants by native gels/zymograms showed that active GOX was produced and secreted. The level of GOX activity correlated with the concentration of glucose in the growth medium, confirming that the *gpd1* promoter is regulated in response to glucose concentration (Fig. 18C). *M. racemosus* R7B transformed with pEUKA4-*gox1* is deposited with DSMZ under Accession number DSM 14068.

The production of secreted GOX during anaerobic yeast growth, aerobic filamentous growth and transition from anaerobic yeast to aerobic filamentous growth was investigated in fermentation experiments. As seen (Fig. 18), active GOX accumulated in culture supernatants both under anaerobic and under aerobic growth conditions. The level of accumulated GOX was higher in the aerobically grown filamentous culture (Fig. 19C) as compared to the anaerobically grown yeast culture (Fig. 19A). However, the highest level of GOX accumulation was observed in the culture which initially had been grown as yeast under anaerobic conditions and then been shifted to aerobic conditions allowing filamentous growth (Fig. 19B). These results indicate that a combination of a yeast growth phase and a filamentous growth phase favors the production and secretion of GOX in *M. racemosus*.

In summary, three *gpd* homologues from the dimorphic zygomycete *Mucor racemosus* have been cloned and characterised. Only expression of *gpd1* was detected and the expression was found to be highly regulated in response to carbon source. The promoter of *gpd1* was characterized and used for recombinant expression of the homologous gene *crgA*, encoding a regulator of carotene biosynthesis, and the heterologous gene *gox1* from *A. niger*, encoding glucose oxidase. Recombinant expression of the regulatory *crgA* gene was evidenced phenotypically by the light independent formation of orange colonies of transformants carrying pEUKA4-*crgA* proving that it is possible to modify the regulation of a metabolic/biosynthetic pathway in *M. racemosus*. Recombinant expression of *A. niger gox1* resulted in production and secretion of enzymatically active GOX.

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#### Example 4

#### **GOX production during dimorphic shift in *Mucor* using a morphogenetically regulated promoter.**

The present example discloses expression of the gene encoding glucose oxidase (GOX) from *Aspergillus niger* (*gox1*, Frederick *et al.*, 1990) directed by a *M. racemosus prnC* promoter.

The plasmid, pEUKA8-*gox1* (Fig. 20), was transformed into *M. racemosus* R7B. *M. racemosus* R7B transformed with pEUKA8-*gox1* is deposited with DSMZ under Accession number DSM 14069.

Expression of *prnC* is induced during aerobic filamentous growth. The production of secreted GOX during anaerobic yeast growth, aerobic filamentous growth and transition from anaerobic yeast to aerobic filamentous growth was investigated in fermentation experiments. As seen in Fig. 21, active GOX accumulated in culture

supernatants both under anaerobic and under aerobic growth conditions. However, the level of accumulated GOX was significantly higher during aerobic filamentous growth (Fig. 21C and 21B after shift from anaerobic to aerobic conditions) as compared to yeast growth under anaerobic condition (Fig. 21A and 21B before the shift from anaerobic to aerobic conditions).

These results indicate that expression of *gox1* under the control of the *pmC* promoter in *M. racemosus* is increased during aerobic filamentous growth, confirming the induction of the *pmC* promoter under these conditions.

## Patent Claims

1. An isolated polynucleotide comprising

- 5           i)       a first nucleotide sequence encoding at least one regulator of morphology capable of regulating the morphology of a dimorphic fungal cell, and operably linked thereto
- 10           ii)       a second nucleotide sequence comprising an expression signal capable of directing the expression of the first nucleotide sequence in a dimorphic fungal cell,

wherein the first and second nucleotide sequences are not natively associated.

15       2. Polynucleotide according to claim 1, wherein the dimorphic fungal cell is capable of growing as a multinucleated cell having a unicellular, essentially spherical morphology and/or capable of growing as a mycelium having a filamentous structure and comprising multinucleated cells.

20       3. Polynucleotide according to claim 2, wherein the multinucleated cells are multipolar.

4. Polynucleotide according to claim 2, wherein the dimorphic fungal cell belongs to the class of Zygomycetes.

25       5. Polynucleotide according to claim 4, wherein the dimorphic fungal cell belongs to the order of Mucorales.

30       6. Polynucleotide according to claim 4, wherein the dimorphic fungal cell belongs to a genus selected from the group of genera consisting of Mucor, Thermomucor, Rhizomucor, Mycotypha, Rhizopus, and Cokeromyces.

7. Polynucleotide according to claim 4, wherein the dimorphic fungal cell belongs to the genus Mucor.

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8. Polynucleotide according to claim 4, wherein the dimorphic fungal cell is a *Mucor* species selected from the group of *Mucor* species consisting of *M. racemosus*, *M. hiemalis*, *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and *M. subtilestissimus*.
- 5 9. Polynucleotide according to claim 8, wherein the *Mucor* species is *M. racemosus*.
- 10 10. Polynucleotide according to claim 1, wherein the dimorphic fungal cell is capable of growing as a uninucleated cell having a unicellular, essentially spherical morphology, and/or capable of growing as a filamentous structure comprising uninucleated cells.
- 15 11. Polynucleotide according to claim 10, wherein the dimorphic fungal cell is selected from the group consisting of *Yarrowia*, *Candida* and *Arxula*.
- 20 12. Polynucleotide according to claim 1, wherein the first nucleotide sequence is selected from the group consisting of
- i) a polynucleotide comprising nucleotides 542 to 1930 of SEQ ID NO:1, and
  - ii) a polynucleotide comprising or essentially consisting of the coding sequence of *pkar* encoding the regulatory subunit of protein kinase A (PKAR) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14062; and
  - iii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and
  - 30 iv) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i), (ii) or (iii), wherein said fragment
    - a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 35



- 5                   b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a cAMP binding domain of *Mucor racemosus* protein kinase A, wherein said cAMP binding domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
- 10                   c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the catalytic subunit for protein kinase A; and
- 15                   v) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) (iii), and (iv), and encodes a polypeptide that
- 20                   a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 25                   b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a cAMP binding domain of *Mucor racemosus* protein kinase A, wherein said cAMP binding domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
- 30                   c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the catalytic subunit for protein kinase A; and
- 35                   vi) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iv) and (v),

and the complementary strand of such a polynucleotide.

13. Polynucleotide according to claim 1, wherein the first nucleotide sequence comprises nucleotides 542 to 1930 of SEQ ID NO:1.
- 5 14. Polynucleotide according to claim 1, wherein the first nucleotide sequence comprises or essentially consists of the coding sequence of *pkar* encoding the regulatory subunit of protein kinase A (PKAR) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14062.
- 10 15. Polynucleotide according to claim 1, wherein the first nucleotide sequence encodes a polypeptide having the amino acid sequence as shown in SEQ ID NO:2.
- 15 16. Polynucleotide according to claim 1, wherein the first nucleotide sequence encodes a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:2, wherein said fragment
- a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
  - 20 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a cAMP binding domain of *Mucor racemosus* protein kinase A, wherein said cAMP binding domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
  - 25 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the catalytic subunit for protein kinase A.
- 30 17. Polynucleotide according to claim 1, wherein the complementary strand of said polynucleotide hybridizes under stringent conditions with the polynucleotide according to any of claims 13 to 16 and encodes a polypeptide that

- a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
  - b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a cAMP binding domain of *Mucor racemosus* protein kinase A, wherein said cAMP binding domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
  - c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to at least one predetermined binding partner, including cAMP and/or the catalytic subunit for protein kinase A.
18. Polynucleotide according to claim 1 and comprising a nucleotide sequence which is degenerate to the first nucleotide sequence according to any of claims 16 and 17.
19. Polynucleotide according to claim 12, said polynucleotide comprising the complementary strand of the polynucleotide according to any of claims 12 to 18.
20. Polynucleotide according to claim 1, wherein the first nucleotide sequence is selected from the group consisting of
- i) a polynucleotide comprising nucleotides 1 to 634 of SEQ ID NO:3, and
  - ii) a polynucleotide comprising or essentially consisting of the coding sequence of *ste20* encoding a MAP kinase kinase kinase (STE20) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14065; and
  - iii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and

- iv) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i), (ii) or (iii), wherein said fragment
- 5 a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 10 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or
- 15 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and
- v) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) (iii), and (iv), and encodes a polypeptide that
- 20 a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 25 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or
- 30 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and
- 35

- vi) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iv) and (v),

5 and the complementary strand of such a polynucleotide.

21. Polynucleotide according to claim 1, wherein the first nucleotide sequence comprises nucleotides 1 to 634 of SEQ ID NO:3.

10 22. Polynucleotide according to claim 1, wherein the first nucleotide sequence comprises or essentially consists of the coding sequence of *ste20* encoding a MAP kinase kinase kinase (STE20) of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14065.

15 23. Polynucleotide according to claim 1, wherein the first nucleotide sequence encodes a polypeptide having the amino acid sequence as shown in SEQ ID NO:4.

20 24. Polynucleotide according to claim 1, wherein the first nucleotide sequence encodes a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:4, wherein said fragment

a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

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b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or

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c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.

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25. Polynucleotide according to claim 1, wherein the complementary strand of said polynucleotide hybridizes under stringent conditions with the polynucleotide according to any of claims 21 to 24 and encodes a polypeptide that

- 5                   a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or
- 10                   c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.
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26. Polynucleotide according to claim 1 and comprising a nucleotide sequence which is degenerate to the first nucleotide sequence according to any of claims 24 and 25.

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27. Polynucleotide according to claim 20, said polynucleotide comprising the complementary strand of the polynucleotide according to any of claims 21 to 26.

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28. Polynucleotide according to claim 1, wherein the first nucleotide sequence is selected from the group consisting of

- 30                   i) a polynucleotide comprising nucleotides 1 to 541 of SEQ ID NO:5, and
- ii) a polynucleotide comprising or essentially consisting of the coding sequence of *mpk1* encoding mitogen activated protein kinase 1 of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14063; and

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- iii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and
- iv) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i), (ii) or (iii), wherein said fragment
- a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or
- c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and
- v) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii), (iii), and (iv), said polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:6, or a fragment thereof, wherein said fragment
- a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or

- c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and

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- vi) a polynucleotide comprising a nucleotide sequence which is degenerate to the nucleotide sequence of a polynucleotide as defined in any of (iv) and (v),

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and the complementary strand of such a polynucleotide.

29. Polynucleotide according to claim 1, wherein the first nucleotide sequence comprises nucleotides 1 to 541 of SEQ ID NO:5.

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30. Polynucleotide according to claim 1, wherein the first nucleotide sequence comprises or essentially consists of the coding sequence of *mpk1* encoding mitogen activated protein kinase 1 of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14063.

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31. Polynucleotide according to claim 1, wherein the first nucleotide sequence encodes a polypeptide having the amino acid sequence as shown in SEQ ID NO:6.

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32. Polynucleotide according to claim 1, wherein the first nucleotide sequence encodes a fragment of the polypeptide having the amino acid sequence as shown in SEQ ID NO:6, wherein said fragment

30

- a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

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- b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or



5 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP; and

10 33. Polynucleotide according to claim 1, wherein the complementary strand of said polynucleotide hybridizes under stringent conditions with the polynucleotide according to any of claims 28 to 32 and encodes a polypeptide that

- 15 a) has Mucor racemosus mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 15 b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of Mucor racemosus mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or
- 20 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.

25 34. Polynucleotide according to claim 1 and comprising a nucleotide sequence which is degenerate to the first nucleotide sequence according to any of claims 32 and 33.

30 35. Polynucleotide according to claim 28, said polynucleotide comprising the complementary strand of the polynucleotide according to any of claims 29 to 34.

36. Polynucleotide according to claim 1, wherein the first nucleotide sequence is selected from the group consisting of

- 35 i) a polynucleotide comprising nucleotides 1 to 384 of SEQ ID NO:7, and

- ii) a polynucleotide comprising or essentially consisting of the coding sequence of *ste12* encoding a transcription factor of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14064; and
- 5      iii) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO:8; and
- iv) a polynucleotide encoding a fragment of a polypeptide encoded by polynucleotides (i), (ii) or (iii), wherein said fragment
  - 10      a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
  - 15      b) is recognised by an antibody, or a binding fragment thereof, which is
    - i) capable of recognising an N-terminal region of STE12 involved in polynucleotide binding and/or ii) capable of recognising an induction domain located in the central region of STE12 and/or iii) capable of recognising a C-terminal region of STE12 involved in transcriptional activation, wherein said recognised region or domain is comprised by
    - 20      the polypeptide having the amino acid sequence as shown in SEQ ID NO:8; and/or
  - 25      c) is competing with a polypeptide having the amino acid sequence as shown in SEQ ID NO:8 for binding to a predetermined binding partner, including a polynucleotide having an affinity for said polypeptide; and
- v) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and
- 30      (iii) and (iv), wherein said fragment
  - a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

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a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or

- 5                   b) is recognised by an antibody, or a binding fragment thereof, which is  
                    i) capable of recognising an N-terminal region of STE12 involved in  
                    polynucleotide binding and/or ii) capable of recognising an induction  
                    domain located in the central region of STE12 and/or iii) capable of  
                    recognising a C-terminal region of STE12 involved in transcriptional  
                    activation, wherein said recognised region or domain is comprised by  
                    the polypeptide having the amino acid sequence as shown in SEQ ID  
                    NO:8; and/or
- 10                  c) is competing with a polypeptide having the amino acid sequence as  
                    shown in SEQ ID NO:8 for binding to a predetermined binding part-  
                    ner, including a polynucleotide having an affinity for said polypeptide.
- 15                  40. Polynucleotide according to claim 1, wherein the complementary strand of said  
                    polynucleotide hybridizes under stringent conditions with the polynucleotide  
                    according to any of claims 37 to 39 and encodes a polypeptide that
- 20                   a) has *Mucor racemosus* STE12 activity and is a regulator of morphol-  
                    ogy of a dimorphic fungal cell; and/or
- 25                   b) is recognised by an antibody, or a binding fragment thereof, which is  
                    i) capable of recognising an N-terminal region of STE12 involved in  
                    polynucleotide binding and/or ii) capable of recognising an induction  
                    domain located in the central region of STE12 and/or iii) capable of  
                    recognising a C-terminal region of STE12 involved in transcriptional  
                    activation, wherein said recognised region or domain is comprised by  
                    the polypeptide having the amino acid sequence as shown in SEQ ID  
                    NO:8; and/or
- 30                   c) is competing with a polypeptide having the amino acid sequence as  
                    shown in SEQ ID NO:8 for binding to a predetermined binding part-  
                    ner, including a polynucleotide having an affinity for said polypeptide.
- 35

41. Polynucleotide according to claim 1, wherein said polynucleotide is degenerate to the first nucleotide sequence according to any of claims 39 and 40.
- 5 42. Polynucleotide according to claim 36, said polynucleotide comprising the complementary strand of the polynucleotide according to any of claims 37 to 41.
43. Polynucleotide according to claim 1, wherein said first and/or second nucleotide sequence is derived from a microbial cell.
- 10 44. Polynucleotide according to claim 43, wherein said microbial cell is selected from the group of microbial cells consisting of eukaryotic microbial cells and procaryotic cells.
- 15 45. Polynucleotide according to claim 44, wherein said microbial cell is a eukaryotic microbial cell.
- 20 46. Polynucleotide according to claim 45, wherein said eukaryotic microbial cell is selected from the group of eukaryotic cells consisting of fungal cells and a yeast cells.
47. Polynucleotide according to claim 46, wherein said eukaryotic microbial cell is a fungal cell, including a filamentous fungal cell.
- 25 48. Polynucleotide according to claim 47, wherein said fungal cell is a dimorphic fungal cell.
49. Polynucleotide according to claim 47, wherein said fungal cell belongs to the class of Zygomycetes.
- 30 50. Polynucleotide according to claim 47, wherein said fungal cell belongs to the order of Mucorales.
- 35 51. Polynucleotide according to claim 47, wherein said fungal cell belongs to the genus selected from the group of genera consisting of Mucor, Mycotypha, and Cokeromyces.

52. Polynucleotide according to claim 47, wherein said fungal cell belongs to the genus *Mucor*.
- 5 53. Polynucleotide according to claim 52, wherein said fungal cell is a *Mucor* species selected from the group of *Mucor* species consisting of *M. racemosus*; *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and *M. subullissimus*.
- 10 54. Polynucleotide according to claim 52, wherein the *Mucor* species is *M. racemosus*.
- 15 55. Polynucleotide according to claim 1, wherein the second nucleotide sequence comprising an expression signal comprises at least one element of a promoter region capable of being regulated, including being induced or repressed, during growth of the dimorphic fungal cell, by any one or more factors including
- 20 a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,
- 25 b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
- 30 c) the growth phase of the dimorphic fungal cell, and
- d) the growth rate of the dimorphic fungal cell.
- 35 56. Polynucleotide according to claim 55, wherein the induction or repression of the expression of the first nucleotide sequence being operably linked to the expression signal is an induction or a repression of said expression, as compared to a predetermined expression level, by at least a factor of 1.02, such as at least a

factor 1.05, for example at least a factor 1.10, such as at least a factor 1.15, for example at least a factor 1.20, such as at least a factor 1.25, for example at least a factor 1.30, such as at least a factor 1.35, for example at least a factor 1.40, such as at least a factor 1.45, for example at least a factor 1.50, such as at least a factor 1.75, for example at least a factor 2.0, such as at least a factor 2.5, for example at least a factor 5.0, such as at least a factor 7.5, for example at least a factor 10, such as at least a factor 15, for example at least a factor 20, such as at least a factor 30, for example at least a factor 40, such as at least a factor 50, for example at least a factor 75, such as at least a factor 100, for example at least a factor 150, such as at least a factor 200, for example at least a factor 500, such as at least a factor 1000, for example at least a factor 2000, such as at least a factor 4000, for example at least a factor 8000, such as at least a factor 10000, for example at least a factor 15000, such as at least a factor 25000, for example at least a factor 50000, such as at least a factor 75000, for example at least a factor 100000.

57. Polynucleotide according to claim 55, wherein the at least one element of the promoter region comprised by the expression signal is regulated, during growth of the dimorphic fungal cell, by the carbon source of the growth medium.

58. Polynucleotide according to claim 55, wherein the at least one element of the promoter region comprised by the expression signal is regulated, during growth of the dimorphic fungal cell, by the oxygen content and the carbon source of the growth medium.

59. Polynucleotide according to claim 55, wherein the expression of the first nucleotide sequence being operably linked to the expression signal comprising the at least one element of the promoter region is induced by the presence in the growth medium, or the addition to the growth medium, of a carbon source.

60. Polynucleotide according to claim 59, wherein the carbon source comprises a hexose

61. Polynucleotide according to claim 60, wherein the carbon source is selected from glucose and galactose.

62. Polynucleotide according to claim 55, wherein the at least one element of the promoter region comprised by the expression signal is selected from the group consisting of

i) a polynucleotide comprising nucleotides 1 to 741 of SEQ ID NO:9,

ii) a polynucleotide comprising or essentially consisting of the promoter region of *gpd1* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14066; and

iii) a polynucleotide comprising at least one fragment of SEQ ID NO:9, wherein said fragment

a) is capable of directing gene expression in a dimorphic fungal cell; and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:9; and

iv) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide

a) is capable of directing gene expression in a dimorphic fungal cell; and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:9,

and the complementary strand of such a polynucleotide.



63. Polynucleotide according to claim 55, wherein the at least one element of the promoter region comprised by the expression signal comprises nucleotides 1 to 741 of SEQ ID NO:9, or a fragment thereof, wherein said fragment is capable of directing gene expression in a dimorphic fungal cell and is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:9.
64. Polynucleotide according to claim 63, wherein said factor is selected from the group consisting of
- a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof
  - b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
  - c) the growth phase of the dimorphic fungal cell, and
  - d) the growth rate of the dimorphic fungal cell.
65. Polynucleotide according to claim 55, wherein the at least one element of the promoter region comprised by the expression signal comprises nucleotides 1 to 741 of SEQ ID NO: 9, or the promoter region of *gpd1* of *M. racemosus*, as deposited with DSMZ under accession number DSM 14066.
66. Polynucleotide according to claim 55, wherein at least one element of the promoter region comprised by the expression signal is selected from the group consisting of
- i) a polynucleotide comprising nucleotides 1 to 755 of SEQ ID NO:10,

ii) a polynucleotide comprising or essentially consisting of the promoter region of *prnC* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14067; and

5 iii) a polynucleotide comprising at least one fragment of SEQ ID NO:10, wherein said fragment

10 a) is capable of directing gene expression in a dimorphic fungal cell; and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 755 of SEQ ID NO:10; and

15 iv) a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide

20 a) is capable of directing gene expression in a dimorphic fungal cell; and/or

25 b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 755 of SEQ ID NO:10,

and the complementary strand of such a polynucleotide.

67. Polynucleotide according to claim 66, wherein the at least one element of the promoter region comprised by the expression signal comprises nucleotides 1 to 755 of SEQ ID NO:10, or a fragment thereof, wherein said fragment is capable of directing gene expression in a dimorphic fungal cell and is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by nucleotides 1 to 755 of SEQ ID NO:10.

68. Polynucleotide according to claim 67, wherein said factor is selected from the group consisting of

- 5                   a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof
- 10                  b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
- 15                  c) the growth phase of the dimorphic fungal cell, and
- d) the growth rate of the dimorphic fungal cell.

69. Polynucleotide according to claim 66, wherein the at least one element of the promoter region comprised by the expression signal comprises nucleotides 1 to 755 of SEQ ID NO: 10, or the promoter region of *prnC* of *M. racemosus*, as deposited with DSMZ under accession number DSM 14067.

70. Polynucleotide according to any of the preceding claims operably linked to a further polynucleotide selected from the group of polynucleotides consisting of a 3' untranslated region, or a fragment thereof, and/or a 5' upstream region, or a fragment thereof.

71. Polynucleotide according to claim 1, wherein the regulator of morphology is a polypeptide capable of regulating gene transcription in a dimorphic fungal cell by forming an interaction with a recognition motif of a promoter region having an affinity for the regulator of morphology.

72. Polynucleotide according to claim 1, wherein the expression of said first polynucleotide results in the production of the regulator in an increased or decreased amount, as compared to the amount of regulator produced, when the

first polynucleotide encoding the regulator is operably linked to the native promoter region under substantially identical growth conditions, and wherein the expression of said first polynucleotide and the production of the regulator in an increased or a decreased amount results in an improved filamentation, or in a dimorphic shift of the dimorphic fungal cell.

73. Polynucleotide according to claim 72, wherein an increased amount of the regulator of morphology is produced from the expression of the first nucleotide sequence encoding said regulator.

74. Polynucleotide according to claim 72, wherein a decreased amount of the regulator of morphology is produced from the expression of the first nucleotide sequence encoding said regulator.

75. Polynucleotide according to claim 72, wherein the amount of regulator produced is increased or decreased at least by a factor of 1.02, such as at least by a factor 1.05, for example at least by a factor 1.10, such as at least a factor 1.15, for example at least by a factor 1.20, such as at least a factor 1.25, for example at least by a factor 1.30, such as at least a factor 1.35, for example at least a factor 1.40, such as at least a factor 1.45, for example at least by a factor 1.50, such as at least by a factor 1.75, for example at least a factor 2.0, such as at least a factor 2.5, for example at least a factor 5.0, such as at least a factor 7.5, for example at least a factor 10, such as at least a factor 15, for example at least a factor 20, such as at least a factor 30, for example at least by a factor 40, such as at least a factor 50, for example at least by a factor 75, such as at least a factor 100, for example at least a factor 150, such as at least a factor 200, for example at least a factor 500.

76. Polynucleotide according to any of claims 72 to 75, wherein the regulator of morphology is a polypeptide encoded by a first polynucleotide comprising a first polynucleotide sequence forming part of a cAMP-dependent signal transduction pathway of a microbial cell, including a fungal cell, including a Mucor species, including M. racemosus,

5 wherein the expression of said first polynucleotide results in the production of the regulator in an increased or decreased amount, as compared to the amount of regulator produced, when the first polynucleotide encoding the regulator is operably linked to the native promoter region under substantially identical growth conditions,

10 wherein the expression of said first polynucleotide and the production of the regulator in an increased or a decreased amount results in an improved filamentation, or in a dimorphic shift of the dimorphic fungal cell.

77. Polynucleotide according to any of claims 72 to 75, wherein the regulator of morphology is a polypeptide encoded by a first polynucleotide comprising a first polynucleotide sequence forming part of a MAP kinase-dependent signal transduction pathway of a microbial cell, including a fungal cell, including a Mucor species, including *M. racemosus*,

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20 wherein the expression of said first polynucleotide results in the production of the regulator in an increased or decreased amount, as compared to the amount of regulator produced, when the first polynucleotide encoding the regulator is operably linked to the native promoter region under substantially identical growth conditions,

25 wherein the expression of said first polynucleotide and the production of the regulator in an increased or a decreased amount results in an improved filamentation, or in a dimorphic shift of the dimorphic fungal cell.

78. Polynucleotide according to claim 72, wherein the dimorphic shift of the dimorphic fungal cell is a shift from a first morphological condition of the dimorphic fungal cell characterised by a unicellular, essentially spherical morphology, to a second morphological condition of the dimorphic fungal cell, wherein the fungal cell comprises a mycelium and is characterised by filamentous growth.

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79. Polynucleotide according to claim 72, wherein the dimorphic shift of the dimorphic fungal cell is a shift from a second morphological condition of the dimorphic fungal cell, wherein the fungal cell comprises a mycelium and is characterised by filamentous growth, to a first morphological condition of the dimorphic fungal cell characterised by a unicellular, essentially spherical morphology.
80. Polynucleotide according to any of claims 78 and 79, wherein the first morphological condition of the fungal cell characterised by a unicellular, essentially spherical morphology is further characterised by an essentially isodiametrical or spherical shape of the fungal cells.
81. Polynucleotide according to any of claims 78 and 79, wherein the second morphological condition of the dimorphic fungal cell, wherein the fungal cell comprises a mycelium and is characterised by filamentous growth, is further characterised by an essentially elongated, hyphal cell shape resulting from a polarised growth of a fungal cell characterised by the first morphological condition.
82. An isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:2, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 2, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide
- a) has *Mucor racemosus* protein kinase A regulatory subunit activity and is a regulator of morphology of a dimorphic fungal cell; and/or
  - b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising a cAMP binding domain of *Mucor racemosus* protein kinase A, wherein said cAMP binding domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:2; and/or
  - c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:2 for binding to

at least one predetermined binding partner, including cAMP and/or the catalytic subunit for protein kinase A.

- 5           83. An isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:4, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 4, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide
- 10                   a) has *Mucor racemosus* STE20 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 15                   b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* STE20, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:4; and/or
- 20                   c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:4 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.
- 25           84. An isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:6, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 6, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide
- 30                   a) has *Mucor racemosus* mitogen activated protein kinase 1 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 35                   b) is recognised by an antibody, or a binding fragment thereof, which is capable of recognising the catalytic domain of *Mucor racemosus* mitogen activated protein kinase 1, wherein said catalytic domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:6; and/or

- 5 c) is competing with a polypeptide comprising or essentially consisting of the amino acid sequence as shown in SEQ ID NO:6 for interaction with a substrate for phosphorylation, including triphosphate nucleotides, including ATP.

10 85. An isolated polypeptide comprising or essentially consisting of the amino acid sequence of SEQ ID NO:8, or a fragment thereof, or a polypeptide functionally equivalent to SEQ ID NO: 8, or a fragment thereof, wherein said fragment or functionally equivalent polypeptide

- 15 a) has *Mucor racemosus* STE12 activity and is a regulator of morphology of a dimorphic fungal cell; and/or
- 20 b) is recognised by an antibody, or a binding fragment thereof, which is i) capable of recognising an N-terminal region of STE12 involved in polynucleotide binding and/or ii) capable of recognising an induction domain located in the central region of STE12 and/or iii) capable of recognising a C-terminal region of STE12 involved in transcriptional activation, wherein said recognised region or domain is comprised by the polypeptide having the amino acid sequence as shown in SEQ ID NO:8; and/or
- 25 c) is competing with a polypeptide having the amino acid sequence as shown in SEQ ID NO:8 for binding to a predetermined binding partner, including a polynucleotide having an affinity for said polypeptide.

30 86. Polypeptide according to any of claims 82 to 85, wherein a substantially identical morphological shift is obtained from the production in a dimorphic fungal cell, under substantially identical conditions, of substantially identical amounts of i) the polypeptide comprising the regulator of morphology of a dimorphic fungal cell, and ii) a functionally equivalent polypeptide comprising a functionally equivalent regulator of morphology, including any fragments thereof.



87. Polypeptide according to any of claims 82 to 85, wherein the functionally equivalent polypeptide, or a fragment thereof, comprises at least one conservative amino acid substitution.

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88. A extrachromosomal, recombinant DNA molecule, preferably in the form of an expression vector, comprising the polynucleotide according to any of claims 1 to 68.

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89. Recombinant DNA molecule according to claim 88 and further comprising a signal sequence encoding a signal peptide, wherein the signal sequence is operably linked to the coding sequence of the first nucleotide sequence.

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90. Recombinant DNA molecule according to claim 88 and further comprising a selectable marker.

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91. Recombinant DNA molecule according to claim 88 and further comprising a genetic element, preferably a transposon, capable of mediating transposition of the recombinant DNA molecule.

92. A fungal host cell transfected or transformed with the polynucleotide according to any of claims 1 to 81, or the vector according to any of claims 88 to 91.

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93. Fungal host cell according to claim 92, wherein said host organism is a dimorphic fungal cell.

94. Fungal cell according to claim 92 or dimorphic fungal cell according to claim 93, wherein said fungal cell or said dimorphic cell further comprises

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i) at least one nucleotide sequence encoding a gene product, and operably linked thereto, and

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ii) at least one further nucleotide sequence comprising a further expression signal capable of directing the expression in a dimorphic fungal cell of the

at least one nucleotide sequence encoding the gene product, wherein said further expression signal is regulatable, during growth of the dimorphic fungal cell, by one or more of

- 5 a) the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,
- 10 b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,
- 15 c) the growth phase of the dimorphic fungal cell, and
- d) the growth rate of the dimorphic fungal cell.

20 wherein the nucleotide sequence encoding the gene product and the further nucleotide sequence comprising the regulatable expression signal are not natively associated.

#### 95. Dimorphic fungal cell comprising

- 25 i) at least one nucleotide sequence encoding a gene product, and operably linked thereto, and
- ii) at least one further nucleotide sequence comprising a further expression signal capable of directing the expression in a dimorphic fungal cell of the
- 30 at least one nucleotide sequence encoding the gene product, wherein said further expression signal is regulatable, during growth of the dimorphic fungal cell, by one or more of
- a) the composition of the growth medium, including at least one of
- 35 carbon source, nitrogen source including amino acids or precursors

thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,

5                   b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,

10                   c) the growth phase of the dimorphic fungal cell, and

d) the growth rate of the dimorphic fungal cell,

15                   wherein the nucleotide sequence encoding the gene product and the further nucleotide sequence comprising the regulatable expression signal are not natively associated.

20                   96. Dimorphic fungal cell according to claim 95 transfected or transformed with the polynucleotide according to any of claims 1 to 81, or the vector according to any of claims 88 to 91.

25                   97. Dimorphic fungal cell according to any of claims 93 to 96, wherein the fungal cell belongs to the class of Zygomycetes.

30                   98. Dimorphic fungal cell according to claim 97, wherein the fungal cell belongs to the order of Mucorales.

35                   99. Dimorphic fungal cell according to claim 97, wherein the fungal cell belongs to a genus selected from the group of genera consisting of Mucor, Thermomucor, Rhizomucor, Mycotypha, Rhizopus, and Cokeromyces.

100.               Dimorphic fungal cell according to claim 99, wherein the fungal cell belongs to the genus Mucor.

101.               Dimorphic fungal cell according to claim 100, wherein the fungal cell belongs to a Mucor species selected from the group of Mucor species consisting

of *M. racemosus*; *M. hiemalis*, *M. rouxii*, *M. genevensis*, *M. bacilliformis*, and *M. substillissimus*.

- 5           102.           Dimorphic fungal cell according to claim 101, wherein the *Mucor*  
species is *M. racemosus*.
- 10           103.           Dimorphic fungal cell according to any of claims 95 to 102, wherein the  
at least one further nucleotide sequence comprising the further expression sig-  
nal is selected from the group consisting of
- 15           i)           a polynucleotide comprising nucleotides 1 to 741 of SEQ ID NO:9,
- ii)           a polynucleotide comprising or essentially consisting of the promoter  
region of *gpd1* of *Mucor racemosus*, as deposited with DSMZ under ac-  
cession number DSM 14066; and
- 20           iii)           a polynucleotide comprising at least one fragment of SEQ ID NO:9,  
wherein said fragment
- a) is capable of directing gene expression in a dimorphic fungal cell;  
and/or
- 25           b) is regulatable, during growth of the dimorphic fungal cell, by at least  
one factor capable of regulating gene expression directed by SEQ ID  
NO:9; and
- 30           iv)           a polynucleotide, the complementary strand of which hybridizes, under  
stringent conditions, with a polynucleotide as defined in any of (i), (ii) and  
(iii), wherein said polynucleotide
- a) is capable of directing gene expression in a dimorphic fungal cell;  
and/or

- b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:9,

5           and the complementary strand of such a polynucleotide.

104.           Dimorphic fungal cell according to any of claims 95 to 101, wherein the at least one further nucleotide sequence comprising the further expression signal is selected from the group consisting of

10

i)           a polynucleotide comprising nucleotides 1 to 755 of SEQ ID NO:10,

ii)           a polynucleotide comprising or essentially consisting of the promoter region of *prnC* of *Mucor racemosus*, as deposited with DSMZ under accession number DSM 14067; and

15

iii)           a polynucleotide comprising at least one fragment of SEQ ID NO:10, wherein said fragment

20

a) is capable of directing gene expression in a dimorphic fungal cell; and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:10; and

25

iv)           a polynucleotide, the complementary strand of which hybridizes, under stringent conditions, with a polynucleotide as defined in any of (i), (ii) and (iii), wherein said polynucleotide

30

a) is capable of directing gene expression in a dimorphic fungal cell; and/or

b) is regulatable, during growth of the dimorphic fungal cell, by at least one factor capable of regulating gene expression directed by SEQ ID NO:10,

5 and the complementary strand of such a polynucleotide.

105. Dimorphic fungal cell according to any of claims 95 to 104, wherein the expression in the dimorphic fungal cell of the nucleotide sequence encoding the gene product results in the production of an increased amount of the gene  
10 product as compared to the production of the gene product in an at least substantially identical dimorphic fungal cell under substantially identical conditions, when the nucleotide sequence encoding the gene product is operably linked to the expression signal natively associated therewith.

15 106. Dimorphic fungal cell according to claim 105, wherein the amount of the gene product is increased by a factor of at least 1.02, for example by a factor of at least 1.05, such as a factor of at least 1.10, for example by a factor of at least 1.15, such as a factor of at least 1.20, for example by a factor of at least 1.25, such as a factor of at least 1.30, for example by a factor of at least 1.35,  
20 such as a factor of at least 1.40, for example by a factor of at least 1.45, such as a factor of at least 1.50.

107. Dimorphic fungal cell according to claim 95, wherein the nucleotide sequence encoding a gene product and/or the further nucleotide sequence  
25 comprising an expression signal is derived from a fungal cell.

108. Dimorphic fungal cell according to claim 95, wherein the gene product is secreted into the growth medium.

30 109. Dimorphic fungal cell according to claim 95, wherein the gene product is selected from the group of gene products consisting of catalase, laccase, phenoloxidase, oxidase, oxidoreductases, cellulase, xylanase, peroxidase, lipase, hydrolase, esterase, cutinase, protease and other proteolytic enzymes, aminopeptidase, carboxypeptidase, phytase, lyase, pectinase and other  
35 pectinolytic enzymes, amylase, glucoamylase, alpha-galactosidase, beta-

galactosidase, alpha-glucosidase, beta-glucosidase, mannosidase, isomerase, invertase, transferase, ribonuclease, chitinase, mutanase and deoxyribonuclease.

5        110.            Method for constructing a recombinant fungal cell according to claim 92, or a recombinant dimorphic fungal cell according to claim 93, said method comprising the step of

10            transforming or transfecting a polynucleotide according to any of claims 1 to 81, or the vector of any of claims 88 to 91, into a fungal cell or a dimorphic fungal cell.

111.            A method of claim 110 and comprising the further step of

15            transforming or transfecting said recombinant fungal cell or said recombinant dimorphic fungal cell with a further polynucleotide comprising

i)            at least one nucleotide sequence encoding a gene product, and operably linked thereto, and

20

ii)           at least one further nucleotide sequence comprising a further expression signal capable of directing the expression in a dimorphic fungal cell of the at least one nucleotide sequence encoding the gene product, wherein said further expression signal is regulatable, during growth of the

25

a)           the composition of the growth medium, including at least one of carbon source, nitrogen source including amino acids or precursors thereof, oxygen content, ionic strength, including NaCl content, pH, low molecular weight compounds, cAMP, and the presence or absence of a cell constituent, or a precursor thereof,

30

b) the temperature of the growth medium, including any change thereof, including an upshift eliciting the expression of one or more heat shock genes,

5 c) the growth phase of the dimorphic fungal cell, and

d) the growth rate of the dimorphic fungal cell,

10 wherein the nucleotide sequence encoding the gene product and the further nucleotide sequence comprising the regulatable expression signal are not natively associated.

112. Method for regulating the morphology of a recombinant fungal cell according to claim 92, or a recombinant dimorphic fungal cell according to any of  
15 claims 93 to 109, said method comprising the steps of

i) cultivating said fungal cell or said dimorphic fungal cell under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and  
20

ii) regulating the morphology of said recombinant fungal cell or said recombinant dimorphic fungal cell.

113. Method for obtaining a predetermined dimorphic shift of a dimorphic  
25 fungal cell according to any of claims 93 to 109, said method comprising the steps of

i) cultivating said dimorphic fungal cell under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and  
30

ii) obtaining a predetermined dimorphic shift of said dimorphic fungal cell, wherein said dimorphic shift results from regulating the expression in said dimorphic cell of said regulator of morphology.

35



114. Method for increasing the filamentation of a dimorphic fungal cell according to any of claims 93 to 109, said method comprising the steps of

- 5 i) cultivating said dimorphic fungal cell under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and
- 10 ii) increasing the filamentation of said dimorphic fungal cell, wherein said increased filamentation results from regulating the expression in said dimorphic cell of said regulator of morphology.

115. Method for increasing the secretory capacity of a dimorphic fungal cell according to any of claims 93 to 109, said method comprising the steps of

- 15 i) cultivating said dimorphic fungal cell under conditions allowing expression of said first nucleotide sequence encoding the at least one regulator of morphology, and
- 20 ii) increasing the secretory capacity of said dimorphic fungal cell, wherein said increased secretory capacity results from regulating the expression in said dimorphic cell of said regulator of morphology.

116. Method for producing a gene product in a dimorphic fungal cell according to any of claims 96 to 109, said method comprising the steps of

- 25 i) cultivating said dimorphic fungal cell under conditions allowing expression of said first nucleotide sequence encoding said at least one regulator of morphology, and
- 30 ii) cultivating said dimorphic fungal cell under conditions allowing expression of said nucleotide sequence encoding said gene product, and
- 35 iii) producing a gene product.

**Abstract**

It is an object of the present invention to provide fungal host organisms capable of expressing recombinant proteins while at the same time exhibiting satisfactory growth characteristics. It is a further object to provide - in a single fungal host organism - the characteristic of homogeneous growth and low viscosity typically associated with yeast organisms, and the capability for high protein secretion normally associated with filamentous fungi. Accordingly, the present invention relates to a recombinant, dimorphic fungal cell comprising a regulatable expression of a regulator of morphology. The nucleotide sequence encoding the regulator of morphology is operably linked to an expression signal not natively associated therewith. Expression of the regulator of morphology directed by the expression signal not natively associated therewith results in a dimorphic shift and/or an improved filamentation of the dimorphic fungal cell. The improved filamentation of the dimorphic fungal cell is positively correlated with an increased production and/or secretion of a desirable polypeptide.

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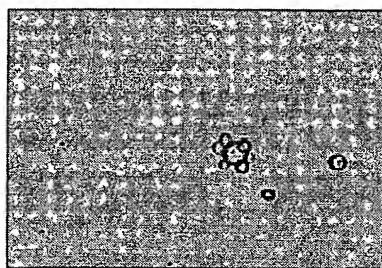
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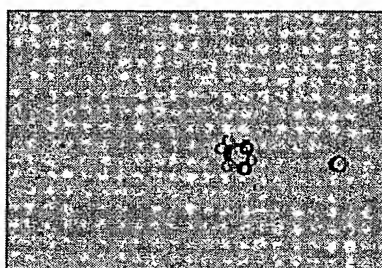
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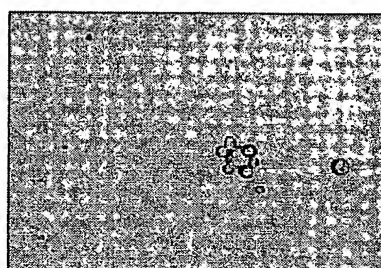
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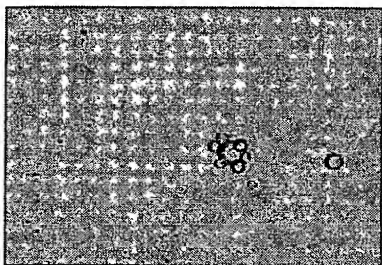
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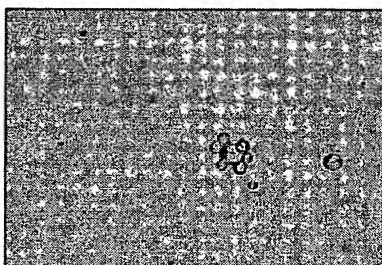
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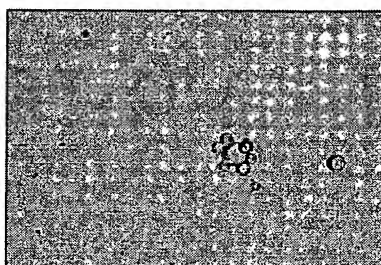
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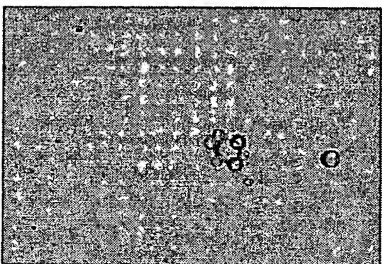
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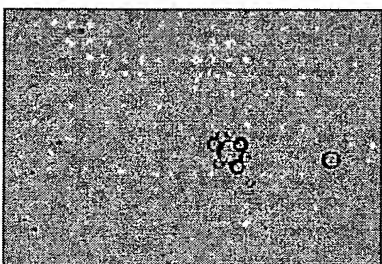
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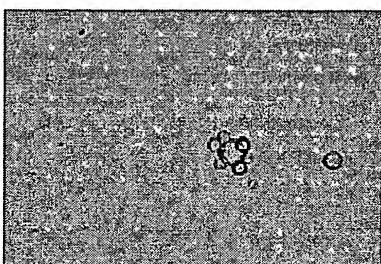
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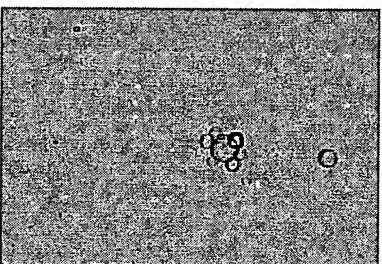
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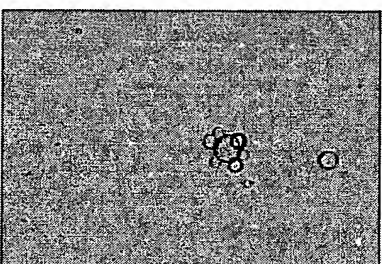
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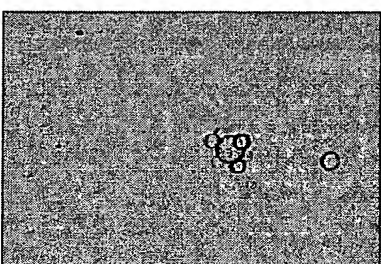
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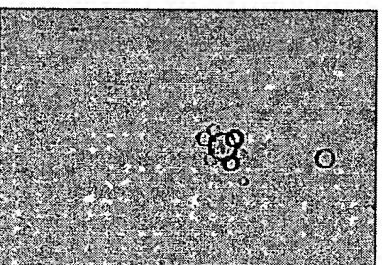
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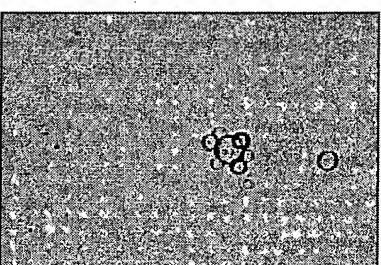
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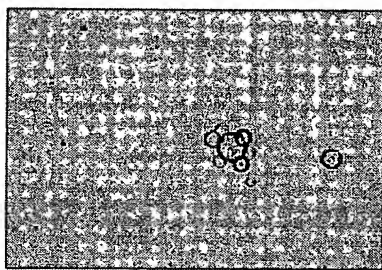
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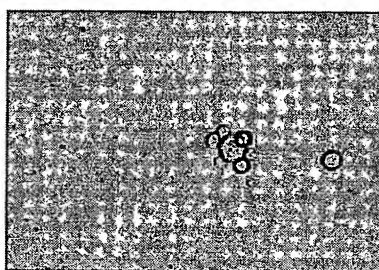
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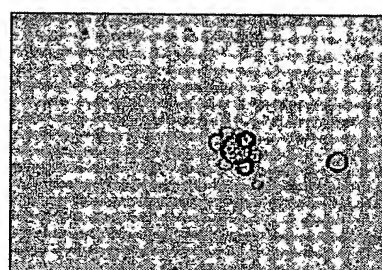




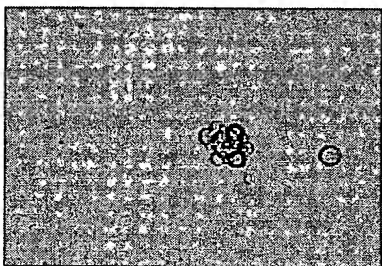
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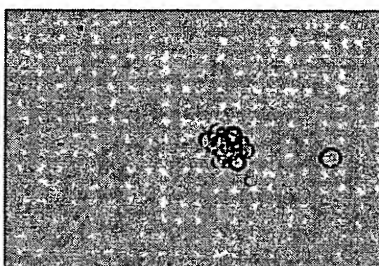
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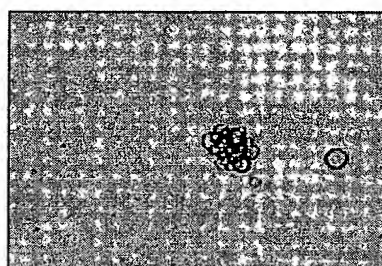
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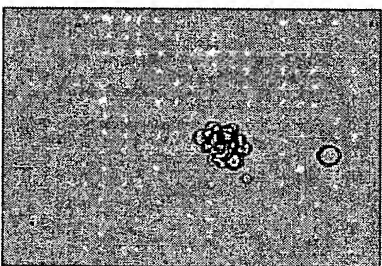
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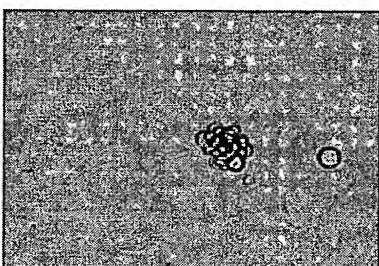
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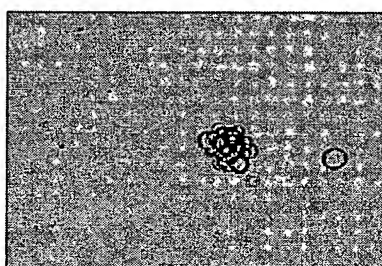
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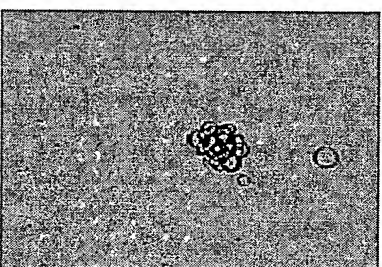
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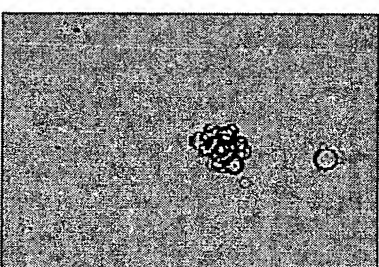
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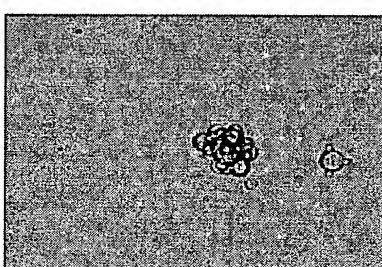
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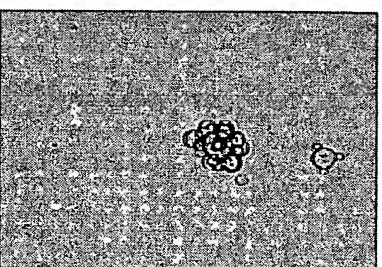
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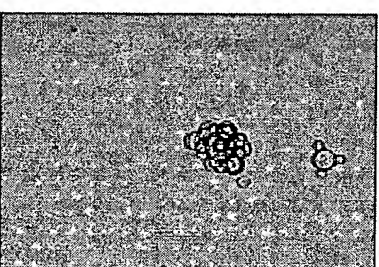
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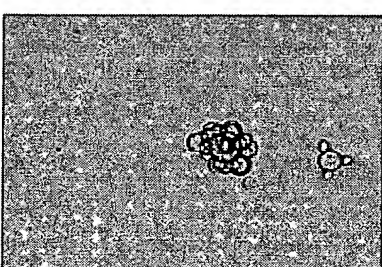
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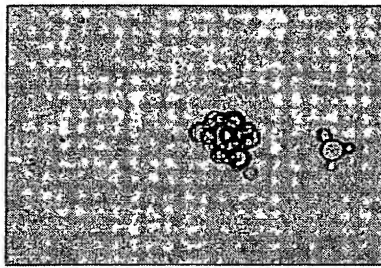


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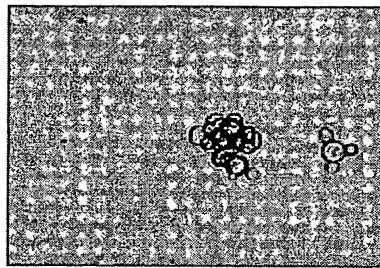


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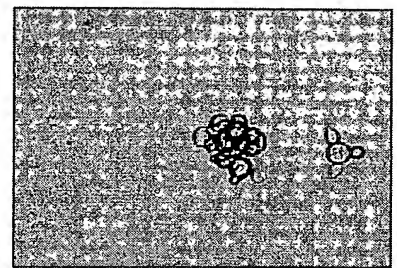
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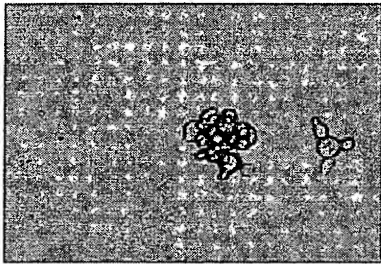
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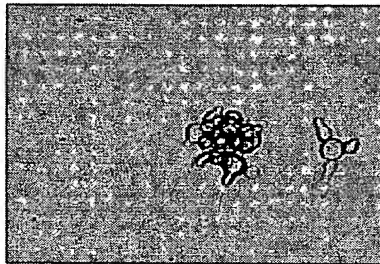
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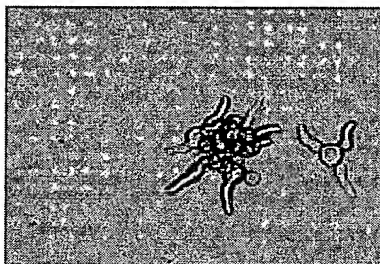
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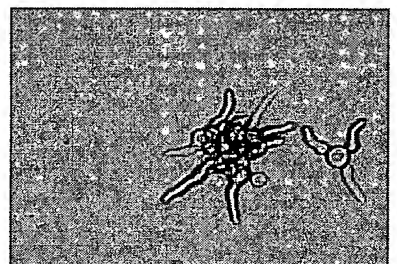
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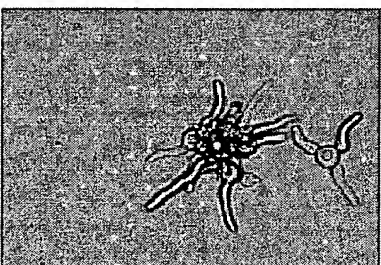
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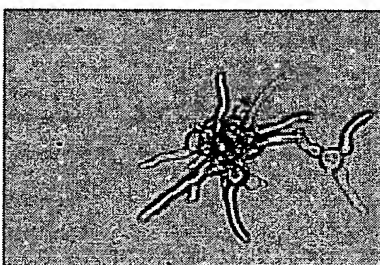
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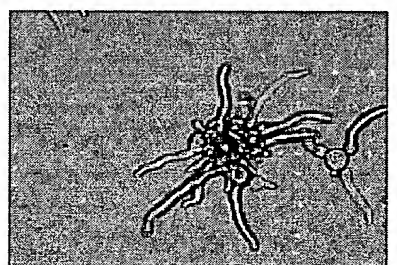
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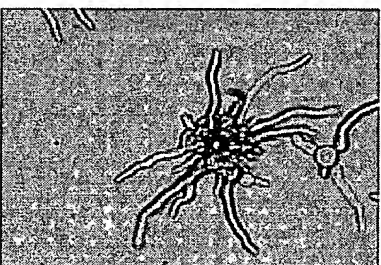
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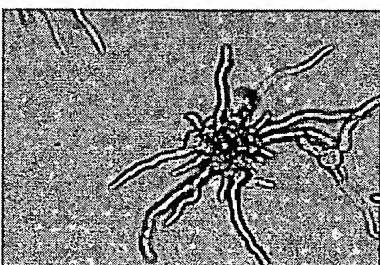
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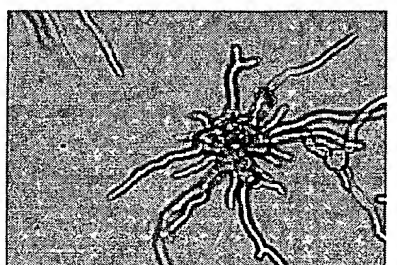
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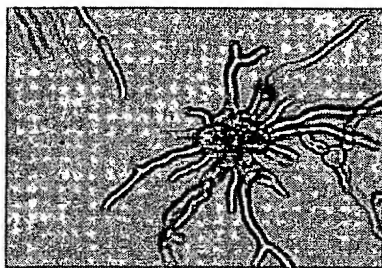
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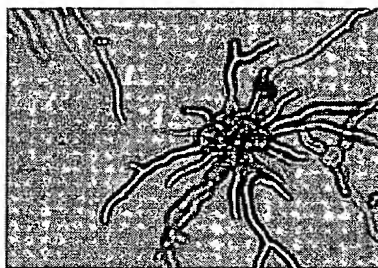
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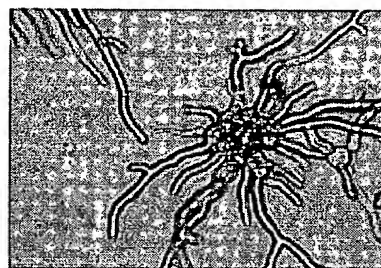




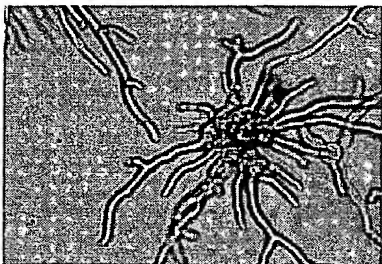
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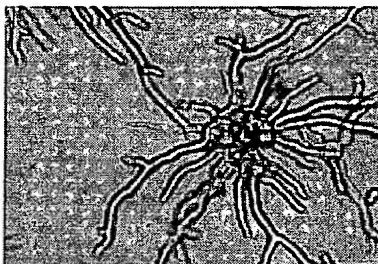
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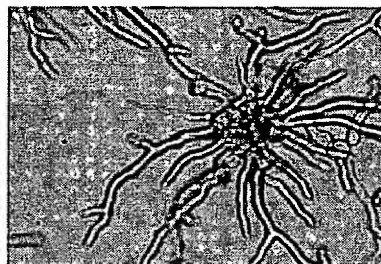
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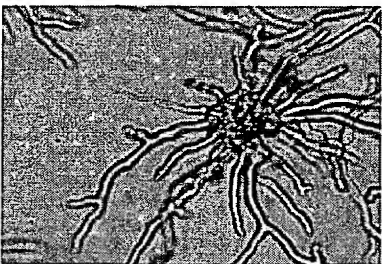
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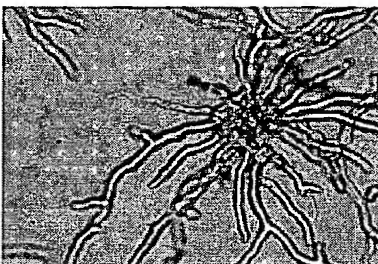
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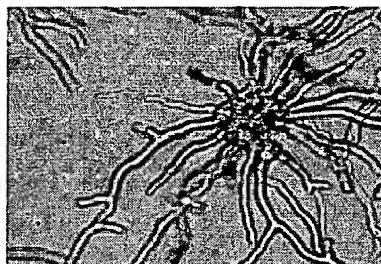
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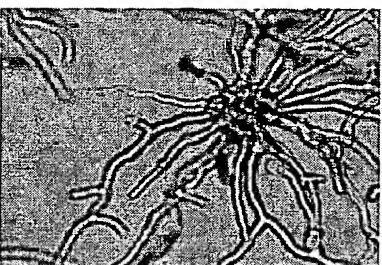
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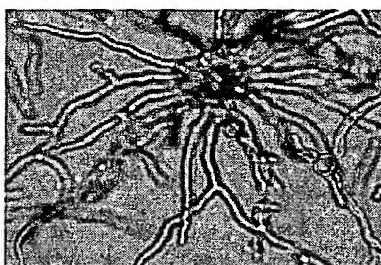
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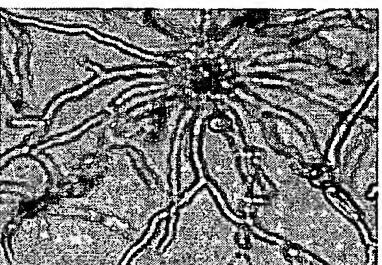
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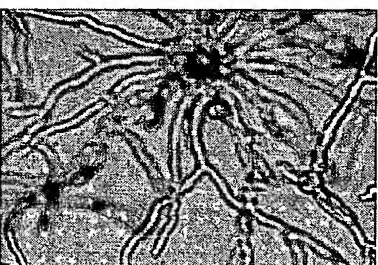
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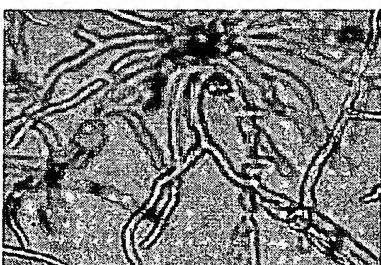
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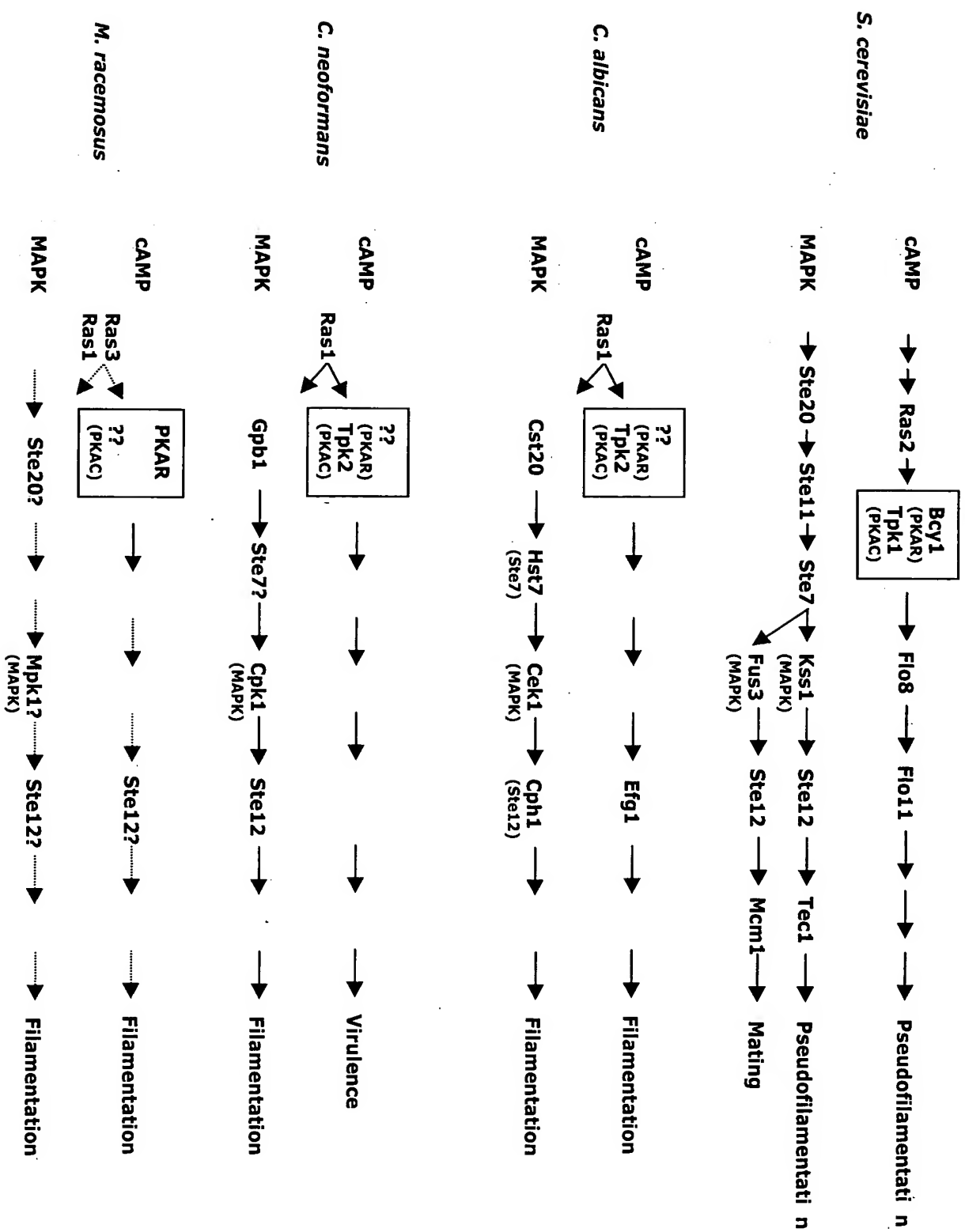


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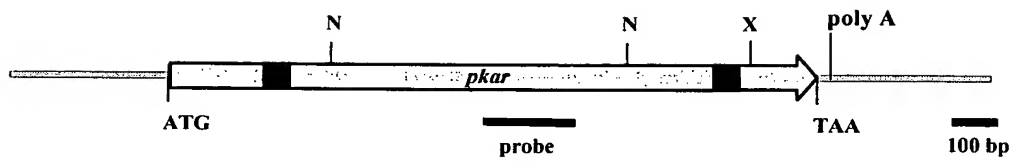
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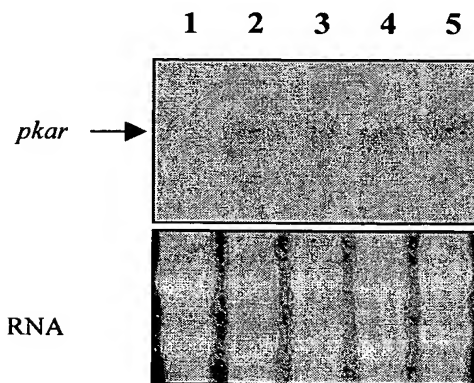


**Fig. 5**

**A**



**B**



**Fig. 6**

```

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1  N-----Mrou
1  MADYTITSLP-----ILKLSHEVLRHQADLVQFCHDYRPIILAQOKVILMSADPATKATIASTAGPAVDADAERANSYAYST----- Beme
1  MVS-SLPKBSQAR-----LQLFCNEINRANPSDFLOHSANYENRLEQQRAELKAREPEFRANKIVLEF-----EFSSFSRPSAQSSQSRSSVMFKS YBCY1
1  MAS--PPACBSEDE-----SLKGGELVQ-----LHGQQVLKICIVHLCISKPERPMHLREHFEKLKEENRQI----- HKAP1

95  -----HPNDHGAL-----HDDDDPLEDEDDREFDKESTEPLESLPTNYNRGRRTSVKCREHG-TQRPRLCOGHQOISQTSERIKVSISNNFLR Mrac
2  -----Mrou
84  -----DGGG-----TEDDDDDDEDD-----AAEPFVNVNRGRRTSVSARSMAPTADVDVAVKIVIPKSDQARARQASIGNNFLR Beme
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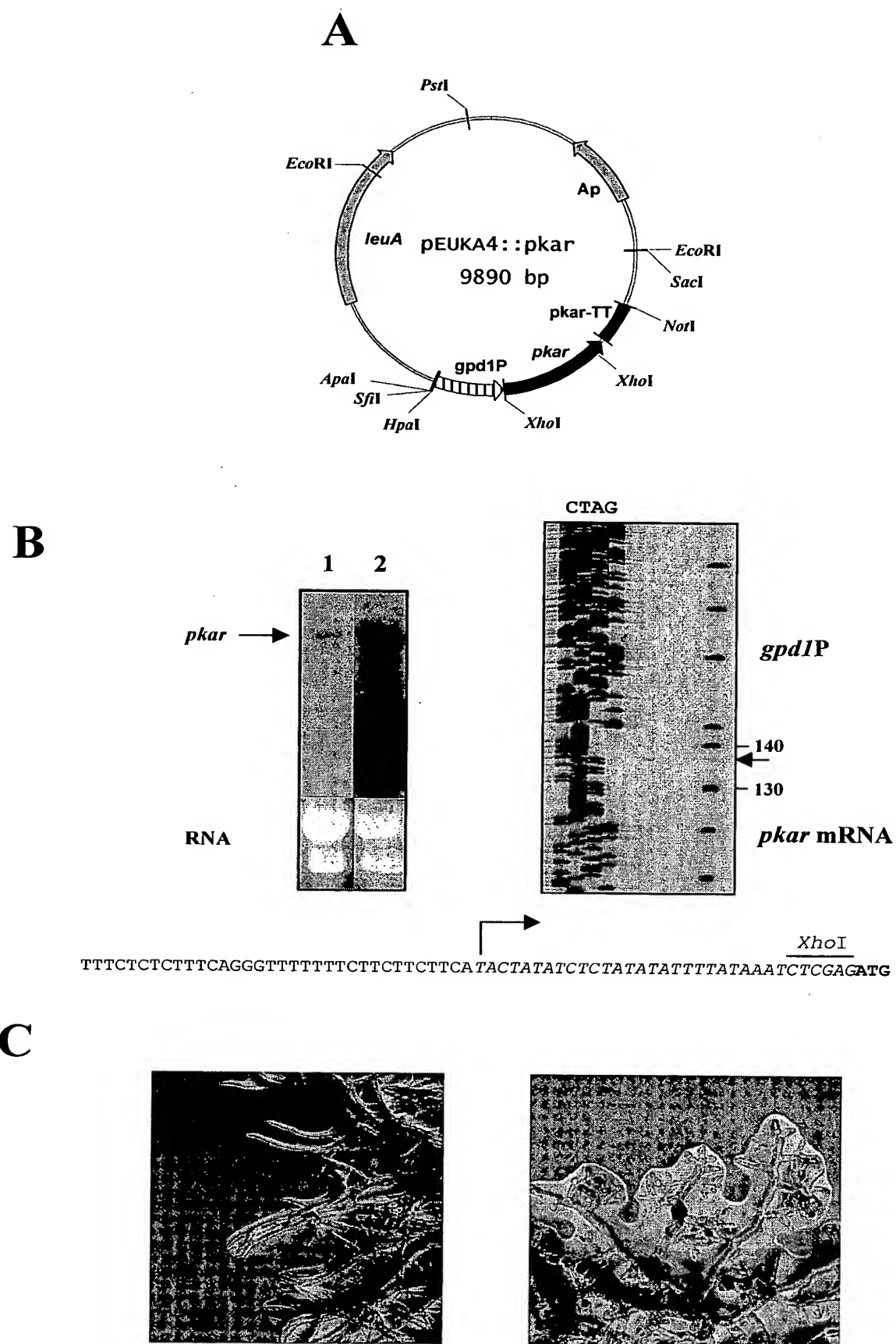
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233 RIIMGSTLRKRMRYBEFLSKVSIIESLWKWEHLTVADRLEHVCFEDGKIVVQGEFGDQFYIITEGTASVLRKSPNBEYVEVGRIGPSDYFGEIALLND HKAP1

373 APRAATVVAHGRLLKCATLGKKAFTRLLGPIVILDKRNSENYHAVINQQS Mrac
198 CPRAATVIAKTLRCVTLGKK Mrou
356 KPRVAILRAKGLKCAELGKKAFTRLLGELADIMQRNTQDYEKYPGEH Beme
375 LPRATVIAATKTHVATLGKSGFORLLGHAVIDVLRINDPT-----RH YBCY1
333 RPRAATVVAHGLKGVKIDRPREHVLGFCSEILKRNIDQYNSFISLTV HKAP1

```

**Fig. 7**



**Fig. 8**

1 -----KFFLATAPVNWENKPLKRFALGGSAAPGG Mrac  
1 MKVQITNSRTEELKQVQANNE---NDEVSKATGEVEESRLIGDLKFFLATAPVNWQENQIRRYIINSG--- Scer  
1 MSITKTNGDPTSLVP-----TQIVKESLRLIEDLKFFLATAPANWQENQVIRRYI----- Calb  
1 MAGSIVKTEIISIS-----GRGDTQSPEEVEESLRLIEDLKFFLATAPANWQENQVIRRYIIS----- Klac  
1 M-----YSQHGAAPPQKPFMFLSNEAQOSLHDAQVALQOVDNLKFLSAPVWQPBQIRRTLE Anid  
1 M-----SEITEEVKESLRLIEDLKFFLATAPANWQENQVIRRYI----- Cpur

34 RSPNGSGESI SCVLWNDFFITGTDIVRSITFREHAFGRVTVNAKKFEEGIFSDLRNLKPGHDARLEEPKSEILMLYKN Mrac  
69 ---GGFVSCVFNNLYYITGTDIVKCLVHMCKFGREVVQKKKFEEGIFSDLRNLKCGIDATLEPKSEFLSFLFN Scer  
53 ---NHDEGFVSCVWNNLYFITGTDIVRCIVMKREHFGKIIDRKKFEEGIFSDLRNLKCGADALEPSEFLSFLFN Calb  
61 ---NDEGFVSCVFNNLYYITGTDIVRCIAYRMCKFGREIYERKKFEEGIFSDLRNLKCGIDATLEPKSFLSFLFN Klac  
66 ---TGDIYSCVWNNLYFITGTDIVRCIAYRMCKFGREIYERKKFEEGIFSDLRNLKCGIDATLEPSEFLSFLFN Anid  
41 ---NDEGFVSCVFNNLYFITGTDIVRCIAYRMCKFGREIYERKKFEEGIFSDLRNLKCGIDATLEPSEFLSFLFN Cpur

114 NCIRTKQKKQVFFWF Mrac  
143 MCLRTQKKQKVFVFWFVADKLFADALERDLKRSSTNQPSSTTKPVNEPALSESYDSSSDKPLYDQIL--QHLDSSRR--PS Scer  
130 SCLRTQKKQKVFVFWFVADKLFADALERDLKRSSTNQPSSTTKPVNEPALSESYDSSSDKPLYDQIL--QHLDSSRR--PS Calb  
137 MCLRTQKKQKVFVFWFVADKLFADALERDLKRSSTNQPSSTTKPVNEPALSESYDSSSDKPLYDQIL--QHLDSSRR--PS Klac  
141 NCIRTKQKKQKVFVFWFVADKLFADALERDLKRSSTNQPSSTTKPVNEPALSESYDSSSDKPLYDQIL--QHLDSSRR--PS Anid  
118 SCLRTQKKQKVFVFWFVADKLFADALERDLKRSSTNQPSSTTKPVNEPALSESYDSSSDKPLYDQIL--QHLDSSRR--PS Cpur

128  
219 STTKSDNSPPKLESENFQNEFVTVINQPLLGVCIMDDDA-----HESFSQINDFIQKLIIEPNTLELNLTL-- Scer  
206 RATSSTNTATTLTDGTVSSGJNNNTISGGGSDATSNNNEASTKP--NGSEKSSBE--YTTTARGRDEFGFLNEATPSQ Calb  
215 PSTVGAQDVDTQKQNNRVITPVSASKQVEHESNVVEVQIVDNNKMCYGLHSESNNYVQQLIVQSDLENNELTNE Klac  
219 ASTTYGQASPVVTVDMAPFQMAPHPSIFILTDESANSQMYT--SIHMHNPFIQ--NLIKREADYQ-----AID Anid  
196 SFRK-----ESTDULASEEFNKKQPF--HGHQNIHQ--E--QEDED--PLD Cpur

128  
287 ---ETPHDLKNT--AKGRDEEDF-----PLDYFEVS--VEYPTENAEDEFPQO---ARTBAAPSMHIS---YDN-- Mrac  
284 MKANSDYEDDFLDYINQTTQNSDYITLDA--NYQAGSYANMIED--NYDSE--LDATLHIEPSLGVTGTAATATS Calb  
295 FQ---ELNADIKPSDIITSNQEDDF-----PLDYFE-----VEIEYSQTSMDSSIHMASQGAHHSQMMFVDEM Klac  
289 YD---RNGMEIARLHQHSSMPTEVEYSPAPSFVSSQVEDYSNRGLSEFPVTEPOHSSHIGEPAYIANEDTGLYTAI Anid  
235 Y-----IDRGVNEHBYISLS--NYMTGRYIN--SHDTNFESIDE--GMFSNP Cpur

128  
346 -----VNERDSMFVNSILN-RYF-----YQLSVAPTFFVHSSSRQHFMTRNDFVSSNNNKEILVSSDSD Mrac  
357 NQVAFNDQVYLIEQAQIR-----TLEPISSSTIGLLQPKSAKFFSLQANGGEEFPAYQNDSTANAGFV--PBI Calb  
357 DGMMLGPKYPIIS-----AGIYEDPF-----FREEMAASNAKYMMEPPIISATBAHFMTRNDFVSSNNNKEILVSSDSD Klac  
364 PEMSTGSGF--NPMMLHPSNIDASAHEFTPARTHSHNVYSVIEGSPYKORRRSS--IPPGAANVTTGTHS--BAP Anid  
277 VNVASNDQVYLIEHTVPIKVGSAVSFSARS-----IDDQSAEF-----PYPAATATGSQF--PPS Cpur

128  
404 PTSYMKYDEBVMDFEERPNECNTNAKSHNSGQQTQHQLYSNNFQOSYPNGMVHGYEYKMPYNPMGGDPLIDQAFYGAD Mrac  
429 SAKYAT--QBATRQVATPTYIKAIPTQGAAGATGNGGQP-----QQYVQATGNAFYF-----AEIHEVLYNVVHES Calb  
422 GRQHGP--DGRRDYDNNVGSPPSENQNPNEELDATEN-----NQDADNRSRDIYF-----BTBAMANDEYIE-- Klac  
436 ALSYAA--HKE-----SDLRSSVSSSVAGDIDSRHESL-----HRVNSTYTATL-----BOKNMMHMSRNGT Anid  
334 AMI--A-QFF-----PSM-----FQASV--B-----VHEHMMHG----- Cpur

128  
484 DFFFPFGCDDNNMLYPQTATSWNVLEPQAMQPAETVGRPYTNYRSTHGSAMFPYMSSNSMQWNTAVSPYSRAPSTT Mrac  
494 EYWT-----NNSGAVATTAANAPMY--DASGFPIE--INQSYMV--MNEHEMVPIQYMNSNGAMI--GM--IE--HHQQOQQO Calb  
485 AYNRRMQMESLVLHETGMMMLNPYMFNCMLAVDS--LNMGVN--VVDFYQGNHSMQVADHDVY--SPQEVVYPNYSRSTF Klac  
495 PLSSVGHREQSSIELTQEDLPALH--ANGTVEG--APNCGVGH--KSRFATGPVRRARSATMM--EL--GPEYQKSHSC Anid  
358 -----NFDVDSYESAGPSAMAVE--SVVHFEPE--PP-----AQFYG----- Cpur

128  
564 AKNPPSTFYSONINQYPRRTVGMKSSQGNVPTGNQSVGSAKISKPLHIRTSAYORQYIN--LETARPSAGDESDA Mrac  
561 QQQI--AMGYQSMLRQOQOQOQOQOQOQ--SSTMTKKKKQIHSHFNNNKSTSSKGGGITKKSHDNNHSHVRTYQSLNJV Calb  
561 KAAV-----FNMRSPYGRNFPSPSAMNPHYTFY-----HRRQPSATRFYFSKANLINRKSSTPPR-----KNVSKPSHK Klac  
568 IPSC--GRIFKRLHLKHLVHTHTQERPYHCPYCNHAFSHSDNIAQHRRFHEAQDQGPLVHEDDLNDDNESVSHDEJE Anid  
392 -----LYDDVEYMRMAVPSQROQE-----ISASMMKKRQOQKTRF--GVSKPKNNRVTEYETRLRQTTIQNL Cpur

128  
643 HEDRNKEISMPFDSNTVVQSEEGGAHSLEVDTNRRSDKN-----IPDAT Mrac  
638 VNSKVTKVINKEEYKQSQTXNNRYREKEKERKKLSKWDNSKFLFVVVYALCLWTCFIMIYF Scer  
627 ARKKVNRTRHAKSHMVGCGKDSGNTSSESDSTKEDSN Klac  
646 SPSESVHE--AVPGVH--GNTSMFSSVAQSTMGSMGSHMA-----POILQQOI Anid  
457 HESHVPTF--B-----SSIVVAS-----QHHS Cpur

Fig. 9



```

1  ---SASNRMPKRVETAEESPSSQTXMDFEIKQEIENR-----Mrac
1  MSNDPSAVSELPAKSLDNGT----SNDNEFAMGGNGDGGGLRTPRTTGTGLNVNAIQKGTNAHEAGGY  Sce
1  MERGTLQRRKRAFNGYGITFVVAHKTEPEVE----VEVEDDLRKIKPRTAPKPPAI--MTNLA-----  Spo
1  MSLSA-AANKISDNDFQNIQEPAPRPSSNSQGRTCYNQTPITKIMSQDLTSAHQ--GTSTSKKKSQW  Calb
1  MAEPSSGSEVHVQISQQAARVIAAAATAPAAATSAAPAPAPAP--AASAAPAPAPAPAPAPAPAAQAVGW  Mou

38  TASAQTVT-DRHILQGYGSSAMVYSVYIPHNKFAIKVIDIMFERNQIDEIRVR-----TALM  Mrac
67  KSMDBAKNAETNDDNNVSLDDPIQFTVSSSSVSGMSSSMSPHINIDEFK---SLFAVTPMINTS  Sce
59  ----EDTFSGRPFSQRTTVSRVSLGS--HQHSSSSIRKIQTNVSDVRSDERNQKSAFENFVSSMSSF  Spo
68  VSYKDDGILSE-IWQKRYLMLHDSYVALYKNDKQNDATIKIPLTSIISVSRTOIKOYCFELVRCSDRNS  Calb
69  --PIGRDAYELQEVIGSGATVAVQAALCKPRQERVAIRINIEKCQTS-MDEL-LKE-----IQAM  Mou

98  ALSKPHVIRVYGSFVHGSKIYITPYMAVGSCLDIMKLSPPDG-----DEISLATILKQALEGLAVI  Mrac
133  NITPDHSAADTFSTINASESDHQFNQ-----ILSKLSLTDSTETIENNTVKHQQFVASSTVNSN  Sce
123  LTGGSSPTSSYGSGSASPRKS-----TVISSPFDPKHVTHVGFNYDTGE  Spo
137  VSGSSSSSLNV--SDSNSRKSIIYIAIKTESDLHSWIDATIAKCPLLSGVSSPTNFTHKVHVGFDPETGS  Calb
126  SQCSHPNVVTYVTSFVVKDEIWLVMKLLSGSMLDIIRYIVNRGEHKNGVIEEATLATILKEVLEGIDVI  Mou

162  HKNGHIHRD--VKAGNIIIMDEGDSVLLAD-----Mrac
194  KSSDTIRATPVSTPVISKPSMTTTEROINSAHSLSNFRHKQHKPKVKPSKPAKSKFVSVKKSFPKSN  Sce
168  FTGMBTEWQALLKVSGITTSQVQHEQAVLDMAFYQSRRVLEEAKPPFPRESTEKLLSVSALSSSS  Spo
205  FVGMETNWEKLLKHSRITGEDWNNSAAVIOVLQEY--QEVNGAGN--ETNTLDKE-----Calb
196  HRNGQIHRD--LKAGNIIIGEDGSVQIADFVSAFLATGGD-----VTBNKVRKTFVGTTPCWMAPE  Mou

189  -----Mrac
264  PKNSPPKKQTEKSYVSS--SSKKRSGNSGTLRMHDFVTSFVQNIKRNQDDKRASSSNNSSSSSI  Sce
238  HLQPTSATSSS--SKLYPSRPAPTPPASSSSSPLISSQIVKTTTSNASRQPSPLVSSKSTDNIIIRHSPV  Spo
257  --QSGETSSSQ--KSI-----PNSVNDNKLNNNSVNSKSSSGV--SSSMVSQRKTSQPPNTKSPV  Calb
255  VMEQVRGYDFKADMWSFGITAEIATGAAPYHKYPPMKVLMILQND--EPTIETGVEDKEMMKKYGKS  Mou

189  -----GVLTK-----Mrac
332  TTAIRISTEYNAKHIHHVGVDS-----KTGEYTGLEDEWEKLITSSGISKEQQQNMQAVMDIVKFYQD  Sce
306  LITPOTITSTETKHIRENNSTHYORRAETSTKPKAVATPQKVEAPAPRLQKRAPEQQSNDSAVIAIRLS  Spo
311  SLGSGSLPEINTKLPTSQSNIE--EHLQNVPNQOYHKMRNGHSPTNQFPFGPMHPNNSQRSLOQQQQQ  Calb
322  FRKILSICLOKDPKRETAELLKCKFFKAKNREYLTBKLLTRTPDIAQRAKKVIRVPGSSGHHTED  Mou

193  -----Mrac
396  VTETNGEDKMFKTENTTTGLPGSP-----QVSTHEANSFNKFPPSTDSDSHNYGSRTG  Sce
376  ICNFRNPTLLVRNEVKIQGASGDVYSARQVGTNLSVAIKKMNINQPKKEFIVNEILVMKSHHKNIVN  Spo
378  QQQQKQHQQYF-YHHQGPSPPSP-SPSPLNYPYPHNMNINPYSKQFOSPLSSQSTQNAIPRYAQNSS  Calb
392  GDWEWSDEENDEKSEEGKAAASQE--KSRVKEENSEISVNAGGIPEQIQSLSVHDSQAGP  Mou

193  -----Mrac
448  --TPM-----Sce
446  FIDTF-----Spo
446  PTAAH-----Calb
450  -----Mou

```

Fig. 11



aagctttcaaagtgtgttgatgaacaattcatcctataatctctaataatgaaatcccgaaga -682  
tctacacagcatcacattcgatagatgggctgctgtttatgtgattaaaacctcactga -622  
tattatctgtttcatgtaaaaaaaactctgttgggtacaaacattagtgtgaaccacg -562  
cgagccataaccactagtcaaaaataatgctctactgcaaaaaatgacgtttgacgaataa -502  
tgcaacgtaaaagtgggttagaaacccttgatatccaaattacacgtgttagcagccttcg -442  
tggttatTTTTcatcacaaactactaggtagctcagggatagttcaaacgggcaatttc -382  
catcctcatcacactttattaccaaggaagaagtgaatggcatcttctatcgttcaa -322  
catctacagggacatctgtgagatacatctgattgctcgacaagcggacaatagatgaca -262  
cgttatcaatgctatcactctaaaatgtcatgtctgactgagtccattgcaatcatcact -202  
ccatccgacatcaggtcacaatttatgcttctatTTTCCAatggatccgaatccgattca -142  
aacaagattaattctccctcaaaaatacccatgaagtgtgagacattgcgaaatgttaTat -82  
aaaCCAatgcattttctcgtctttcaggggtttttttcttcttcttcaTactatatctcta -22  
tatattttataaattctaacaATGGTTGTTCAAGTCGGTATTAACGGTTTCGGTCGTATT 39  
M V V Q V G I N G F G R I  
GGTCGTATTGTCTTCGTGCTACTGAGTCCAACAAGGATGTCCAAGTTGTTGCTATCAAC 99  
G R I V L R A T E S N K D V Q V V A I N  
GATCCCTTCATTCTCTCGACTATATGGTCTACATGTTGAAGTACGATACTGTTACCGGT 159  
D P F I P L D Y M V Y M L K Y D T V H G  
CGTTTCGATGGTTCCGTCGAGGCCAAGGATGGTAAGCTCGTTGTCAACGGTCATGCTATC 219  
R F D G S V E A K D G K L V V N G H A I  
GCCGTCTCTGCTGAGCGCGATCCTACCTCTATTCTTGGGGTTCCGCTGGTGCTGACTAC 279  
A V S A E R D P T S I P W G S A G A D Y  
GTTGTCGAGTCCACTGGgtaaatataactgaaatgcattatatctgaatatctaatactaa 339  
V V E S T G intron1  
cattgacgtaatatGTCTTCACTACCACTGAAGCTGCCCTCTGCTCATCTTAAGGGTGGT 399  
V F T T T E A A S A H L K G G  
GCCAAGAAGGTCATCATCTCTGCTCCCTCTGCTGATGCCCCCATGTTTCGTCTGTGGTGTC 459  
A K K V I I S A P S A D A P M F V C G V  
AACCTCGAAGCTTACAAGTCTGAATACAAGGTTATCTCCAACGCCTCTTGTACCACCAAC 519  
N L E A Y K S E Y K V I S N A S C T T N  
TGTTTGGCTCCCCTCGCCAAGGTCATTAACGATAACTTTGGTATCGCTGATGGTTTGTATG 579  
C L A P L A K V I N D N F G I A D G L M  
ACCACTGTCCACGCAACCACTGCCACCCAAAAGACTGTCGATGGTCCCTCTCACAAGGAT 639  
T T V H A T T A T Q K T V D G P S H K D  
TGGAGAGGTGGTCTGCGCTGCTGCCAACATCATCCCCTCTTCCACTGGTGCTGCCAAG 699  
W R G G R A A A A N I I P S S T G A A K  
GCTGTCGGTAAGGTCATTCCCGCTCTCAACGGTAAGCTCACTGGTATGGCTTTCCGTGTC 759  
A V G K V I P A L N G K L T G M A F R V  
CCCACCCCGATGTCTCTGTGCTGATTGACCGTCAACCTCTCCAAGGGTGCTTCTTAT 819  
P T P D V S V V D L T V N L S K G A S Y  
GATGAAATCAAGCAAGCCATCAAGAAGGCCTCTGAACTACCATGAAGGGTGTCTCGGT 879  
D E I K Q A I K K A S E T T M K G V L G  
TACACTTCTGATGCTGTTGTGTCAGCAGTGATTTCGTGGGTGAAGTTTGgtaagaaacgtta 939  
Y T S D A V V S S D F V G E V C  
ttattttcatcgtttgaatagttttactaaccattgaaaatcatagTTCTTCCGTATTTGACG 999  
intron2 S S V F D  
CTGCTGCCGGTATCCAATTGACCCCCACTTTTGTTAAGCTTATCGCTTGGTATGACAATG 1059  
A A A G I Q L T P T F V K L I A W Y D N  
AGTATGGTTACTCTAACCGTGTGCTTGACCTCCTCGTTCATGCCGCTAAGGTCGATGGTG 1119  
E Y G Y S N R V V D L L V H A A K V D G  
CTCTCTAAatcgtaaatcattttctagtcatttgcatttcatacacacatctgttacataaa 1179  
A L .  
taaaattcatgtaaaaagtgcggtcataagatcgttttttgttaattagcttatattaatt 1239  
tctgttccaaccctctgatatgtaaaatgttgacgaattgcaagtattttgacaggcaga 1299  
atgacagcatatatttgaxgcctgtgvacaatctgtgttacataagattcctggtaaagg 1359  
atggatgatattatattttacagttataagagccggtattggcacacgaaggaagccttg 1419  
cagcgagaaggacgacgctcttttttataggctcatcactcaatgagagttgcaggaagc 1479  
actattttgtaaagtcctgaaatacagagaccctctggactattattctcaagaagcact 1539  
ttaacaagaaaaatatagttcttttgctaatttcaagaccttaatcatatatxxcgcttt 1599  
catttttatttcattggtttcattcaattttatagatgtattactacactactgattgctgt 1659  
tactgttactatcgccctggccattgtgtgtgtgtgtcgctgccatgcacatcgccgtt 1719  
attgtcatcgc 1730

Fig. 12

gcgccatctactgctcttgccatgcaaagtgggtacgggtgcattatccaaactttgtgct -290  
 ctatgtataacatcatttactgctgctgcccctactctttcaactatatttagtgcgtca -230  
 cagaaagatctgagtcataatatagtaaaaaatttaaaggactgggaagctactcatgtg -170  
 caactcaagaaaacgcaagttacaccaaacac**ccaatt**tttaaaacagcatagtcgctctgtt -110  
 gattttatcttgattaatccccaaaaaaag**tataaaa**agttgatggattttcatggacgcg -50  
 tctcttcttcttcttctttttgttttcacattaaataaattctatataaaaaATGGTTACTCA 11  
 M V T Q  
 AGTTGGTATTAACGGGtaagttaaagctgaattgatttgaacatttgatgttcctgaat 71  
 V G I N G *intron1*  
 tggctcacctcacacatcccaatggtttataatagTTTCGGCCGTATTGGTCGTATTGTTC 131  
 F G R I G R I V  
 TCCGTGCTTCTCTCTCTAACCCTGAAGTCCAAGTTGTTGCTATCAACGATCCCTTCATCC 191  
 L R A S L S N P E V Q V V A I N D P F I  
 CCTTGGAATACATGGTCTACATGTTCAAGTACGATTCCGTTACGGTCGTTTCCAAGGTA 251  
 P L E Y M V Y M F K Y D S V H G R F Q G  
 CTGTTGAGGCCAAGGATGGTAAGCTCGTTGTCAACGGTAAGGAGATCTCCGTCTTCTCTG 311  
 T V E A K D G K L V V N G K E I S V F S  
 AGCGTGACCCTGCTCAAATCCCTTGGGGTTCCGTTGAAGCTGCCTATGTTGtTGAGTCTA 371  
 E R D P A Q I P W G S V E A A Y V V E S  
 Ctgaagtatacgcgcaatcattccccgagaaaaatgcaatg**ttaac**attggacaccagGG 431  
 T *intron2* G  
 TGTCTTCACCTCTATTGATGCTGCTTCTGCCCATCTTCAAGGTGGTGCCAAGAAGGTTAT 491  
 V F T S I D A A S A H L Q G G A K K V I  
 CATCTCTGCTCCTTCTGGTGATGCTCCCATGTTTGTCTGTGGTGTCAACCTCGAGAAGTA 551  
 I S A P S G D A P M F V C G V N L E K Y  
 CACCTCTGACCTCAAGGTCATCTCCAACGCCCTCTGTACCACCAACTGTTTgGCTCCCT 611  
 T S D L K V I S N A S C T T N C L A P L  
 TGCCAAGGTATCAATGACAACCTTGGTATCGTTGAAGGTTTGATGACCACTGTCCATGC 671  
 A K V I N D N F G I V E G L M T T V H A  
 TACTACTGCTACCCAAAAGACTGTGCGATGGTCTTCCAACAAGGACTGGCGTGGCGGTCG 731  
 T T A T Q K T V D G P S N K D W R G G R  
 TGGTGCTGGTGCCAACATCATCCCTCTTCCACTGGTGCTGCCAAGGCTGTGCGTAAGGT 791  
 G A G A N I I P S S T G A A K A V G K V  
 CATTCCTGAACCTTAACGGTAAGCTCACTGGTATGGCTTCCGTGTCCCTACCCCTGATGT 851  
 I P E L N G K L T G M A F R V P T P D V  
 CTCTGTGCTTGATCTTACTGTGAGATTGGAGAAGgtaaataggtctgcttggttatatttg 911  
 S V V D L T V R L E K  
 gagacaaaagctgacattttgtttgtattcattagGGTGCTACCTACGAAGAAATCAAGG 971  
*intron3* G A T Y E E I K  
 CTGTTATCAAGAAGGCCTCTGAGAACGAGTTGAAGGGTATCCTCGGTTATACCAACGACC 1031  
 A V I K K A S E N E L K G I L G Y T N D  
 AAGTTGTAAGCACCGATTTCGTGGGTGATGCTCAAAGCTCCATCTTCGATGCTGCTGCTG 1091  
 Q V V S T D F V G D A Q S S I F D A A A  
 GTATTGCTCTTAACGACAAGTTCGTCAAGCTCGTCTCTTGGTACGATAACGAATTCCGTT 1151  
 G I A L N D K F V K L V S W Y D N E F G  
 ACTCCAACCGTGTGATCGATCTTTTGGCCTATGCTGCCAAGGTTGATGCTGCTGCTCAAT 1211  
 Y S N R V I D L L A Y A A K V D A A A Q  
 Aaatgtattgacaatgcctatatcttgtttcatattaaaaatttctcaattgttttataa 1271  
 .  
**ataaaa**atctctcgaacttacatacaaaaactcatcgctctctgctacattgctggcatgttc 1331  
 ttctggtcggatatatctttatcagtcatttactgatcttcacatgtaaatgaatatag 1391  
 acgtcttttacaatgtacatgtcggagacaaaattgctcgttccgtatatttgct 1446

Fig. 13

cgtaacgtattacaacatatcccttcagaaaaaaagta[tataaa]cctcgacattctttgt -320  
 ctcgagttttgttttcctcataaccaaaacaacccaattttttttttccttagttatcaac -260  
 taacgaaaaaaaaccctcaagcaaatctatacaacaactttttttaactttttttaaac -200  
 ttttaacgaccccatTTTTTcatTTTtaacgaaaaaaactctttcatattactTTTtacat -140  
 ttctactctactcacgaaatctaaaaagcactTTTtaacgaaccatTTTtaacgaaaaaac -80  
 tcaatttttacgaccccaaaaaaacacattTTTtaactttaccctTTTTTTTaaatctcttt -20  
 aaaacaaattttcttcaatATGTCTACTATCAACACTGGTATTAACGGgtaacgtatataca 41  
 M S T I N T G I N G  
 aggagcctcctgggcaggacctggaaacaaaaaactaacaatgcatatcctctagTTTCGG 101  
 intron1 F G  
 TCGCATTGGCCGTATTGTCTTGCGTGCCTCCCTCGAAAACCCCAAGGTCAAGGTGCGTCGC 161  
 R I G R I V L R A S L E N P K V K V V A  
 TATCAATGATCCCTTCATTGATCTTGAATACATGGTCTACATGTTCAAGTATGATTCCAC 221  
 I N D P F I D L E Y M V Y M F K Y D S T  
 TCACGGCCGATTCAAGGGCACTGTCGAGCACAAGGACGGTAAACTGGTAGTCAATGGCCA 281  
 H G R F K G T V E H K D G K L V V N G H  
 CGAAATCGCAGTTCATGCCGAACGTGATCCAGCTCAAATCCCCTGGGGTTCTCATGGCGC 341  
 E I A V H A E R D P A Q I P W G S H G A  
 TGATTATGTTATCGAATCGACTGGTGTATTCACTACCAAGGACGCTGCTTCTGCTCATCT 401  
 D Y V I E S T G V F T T K D A A S A H L  
 CAAAGGTGGGGCGAAAAGGTTATCATTTCAGCCCCTTCTGCGGATGCCCcTATGTTTGT 461  
 K G G A K K V I I S A P S A D A P M F V  
 TGTAGGCGTAAATCTTGACAAGTATACGTCTGATCTCACCGTCATCTCCAACGCCTCGTG 521  
 V G V N L D K Y T S D L T V I S N A S C  
 TACCACGAATTGTCTCGCTCCTTTGGCGAAAGTCATTACGACAATTATGGTATCTTGGGA 581  
 T T N C L A P L A K V I H D N Y G I L E  
 AGGTCTAATGACAACGTTCATGCTACAACCTGCCACTCAAAAAACGGTCGATGGTCCATC 641  
 G L M T T V H A T T A T Q K T V D G P S  
 CAACAAGGACTGGAGAGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG 701  
 N K D W R G G R G A G A N I I P S S T G  
 CGCGGCGAAAGCTGTTGGCAAAGTTATCCCTGAACTCAATGGAAAACCTCACTGGCATGGC 761  
 A A K A V G K V I P E L N G K L T G M A  
 TTTCAGAGTACCTACCCCTGATGTTTCTGTGGTTGATTAACTGTGCGACTGGAAAAGgt 821  
 F R V P T P D V S V V D L T V R L E K  
 acaatagtaatggggttacatttgattgtacaacagcatgctaacttttatatatattc 881  
 intron2  
 aatagCCCGCATCGTATGATGAAATCAAAGCTACCATCAAGAAGGCCTCTGAAAGTGAGG 941  
 P A S Y D E I K A T I K K A S E S E  
 AACTCAAGGGTATCTTAGGATACACCGAGGACCAAGTGGTGTGCGACCGATTTTGTGGGAG 1001  
 E L K G I L G Y T E D Q V V S T D F V G  
 ATGCGCACTCTAGCATCTTTGATGCAAAGGCAGGTATTCAACTATCTCCAACATTTGTCA 1061  
 D A H S S I F D A K A G I Q L S P T F V  
 AGCTCATCAGTTGGTACGACAATGAATTTGGCTATTCAACAAGAGTGGTGGATCTATTGG 1121  
 K L I S W Y D N E F G Y S T R V V D L L  
 CTTATGTTGCTGGTAAAGATGGTGGTCTGTAAattttaacccttacattccccctcttct 1181  
 A Y V A G K D G A L  
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 aaaaaatcaaacatacccaacacactccttctctctctcgcttccctttctcactcctcat 1361  
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 atgtgattggaatcttttgatccaaaccaagatagccatcactgatcaggtacaaggtgt 1601  
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Fig. 14

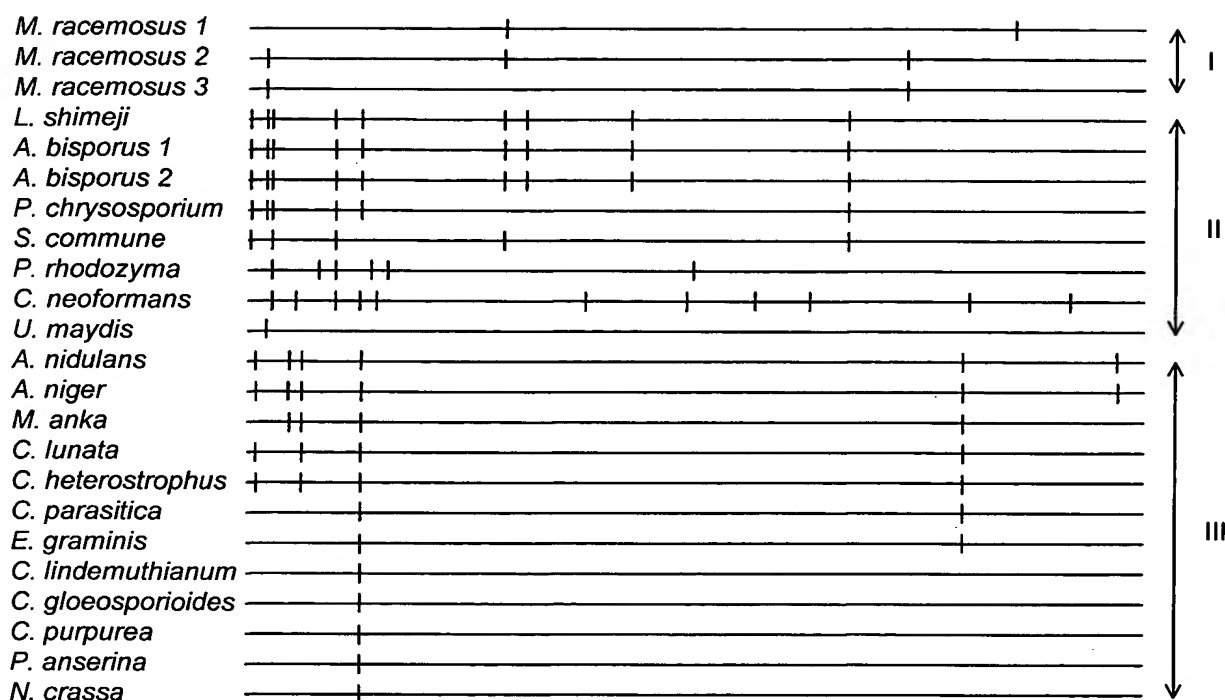


Fig. 15A

gpd1 1 M S T I N T G I N G F G R I G R I V L R A S L E N P K V K V V A I N D P F I D L E Y M V Y M F K Y D  
gpd2 1 M V T - Q V G I N G F G R I G R I V L R A S L S N P E V Q V V A I N D P F I P L E Y M V Y M F K Y D  
gpd3 1 M V - V Q V G I N G F G R I G R I V L R A T E S N K D V Q V V A I N D P F I P L D Y M V Y M L K Y D

51 S T H G R F K G T V E H K D G K L V V N G H E I A V H A E R D P A Q I P W G S H G A D Y V I E S T G  
50 S V H G R F Q G T V E A K D G K L V V N G K E I S V F S E R D P A Q I P W G S V E A A Y V V E S T G  
50 T V H G R F D G S V E A K D G K L V V N G H A I A V S A E R D P T S I P W G S A G A D Y V V E S T G

101 V F T T K D A A S A H L K G G A K K V I I S A P S A D A P M F V V G V N L D K Y T S D L T V I S N A  
100 V F T S I D A A S A H L Q G G A K K V I I S A P S G D A P M F V C G V N L E K Y T S D L K V I S N A  
100 V F T T T E A A S A H L K G G A K K V I I S A P S A D A P M F V C G V N L E A Y K S E Y K V I S N A

151 S C T T N C L A P L A K V I H D N Y G I L E G L M T T V H A T T A T Q K T V D G P S N K D W R G G R  
150 S C T T N C L A P L A K V I N D N F G I V E G L M T T V H A T T A T Q K T V D G P S N K D W R G G R  
150 S C T T N C L A P L A K V I N D N F G I A D G L M T T V H A T T A T Q K T V D G P S H K D W R G G R

201 G A G A N I I P S S T G A A K A V G K V I P E L N G K L T G M A F R V P T P D V S V V D L T V R L E  
200 G A G A N I I P S S T G A A K A V G K V I P E L N G K L T G M A F R V P T P D V S V V D L T V R L E  
200 A A A N I I P S S T G A A K A V G K V I P A L N G K L T G M A F R V P T P D V S V V D L T V N L S

251 R P A S Y D E I K A T I K K A S E S E E L K G I L G Y T E D Q V V S T D F V G D A H S S I F D A K A  
250 K G A T Y E I K A V I K K A S E N E - L K G I L G Y T N D Q V V S T D F V G D A Q S S I F D A A A  
250 K G A S Y D E I K Q A I K K A S E T T - M K G V L G Y T S D A V V S S D F V G E V C S S V F D A A A

301 G I Q L S P T F V K L I S W Y D N E F G Y S T R V V D L L A Y V A G K D G A L .  
299 G I A L N D K F V K L V S W Y D N E F G Y S N R V I D L L A Y A A K V D A A A Q .  
299 G I Q L T P T F V K L I A W Y D N E Y G Y S N R V V D L L V H A A K V D G A L .

Fig. 15B

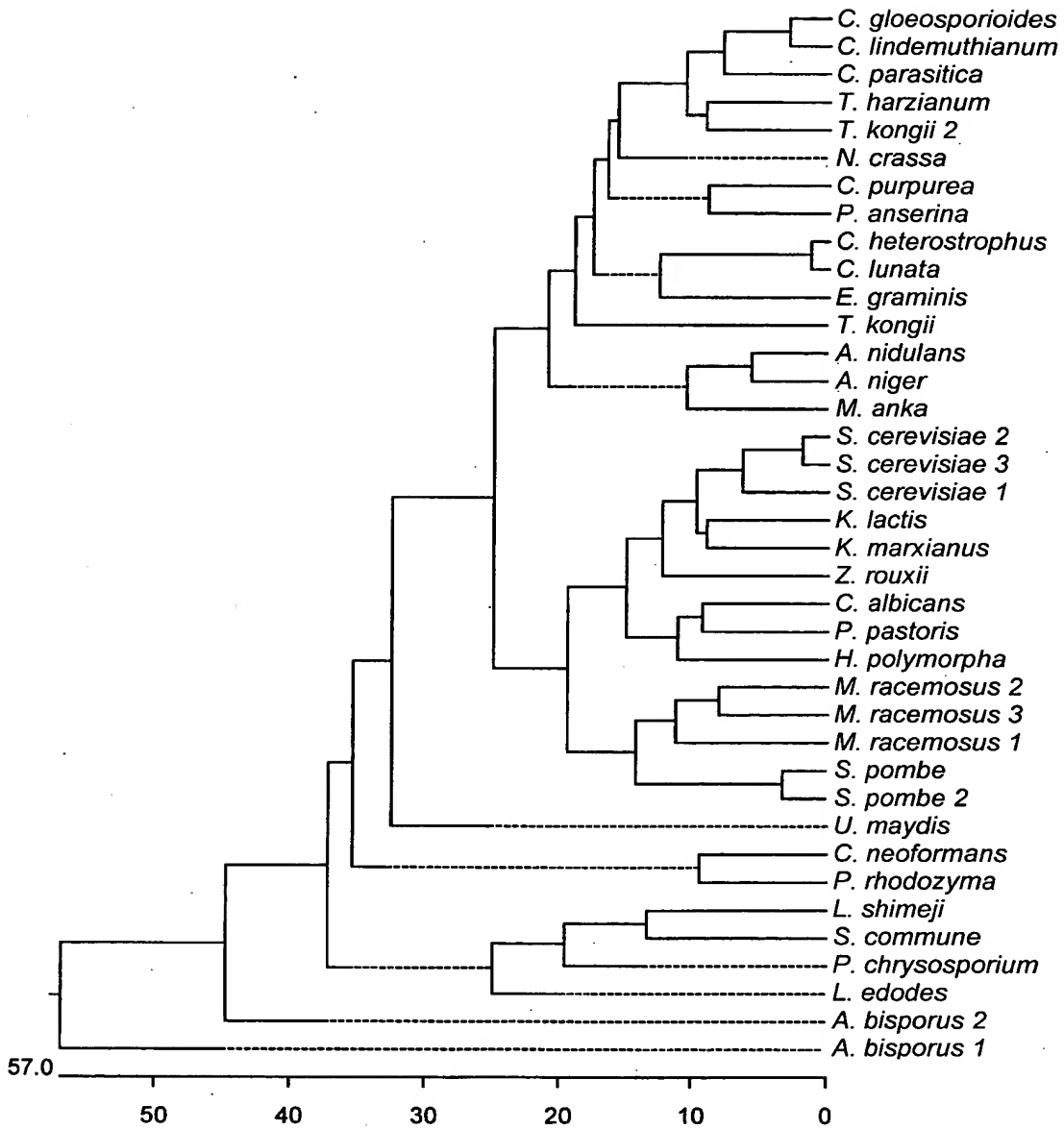


Fig. 15C

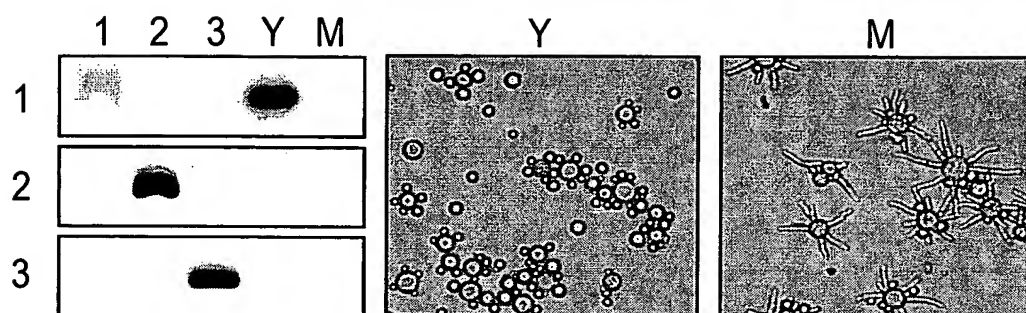


Fig 16

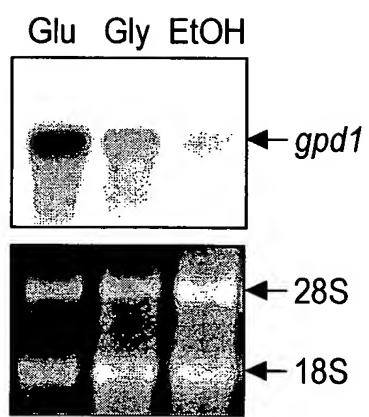


Fig. 17A

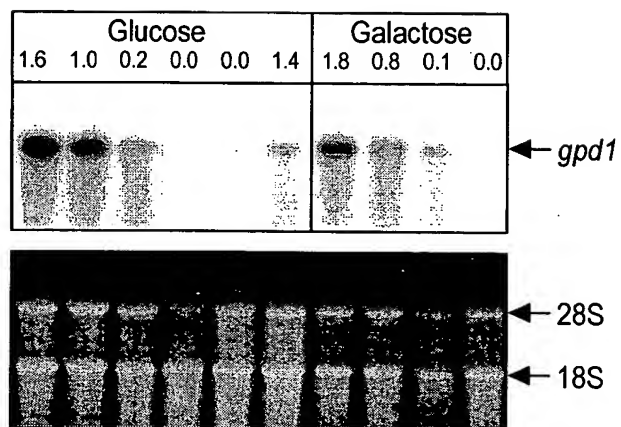


Fig. 17B

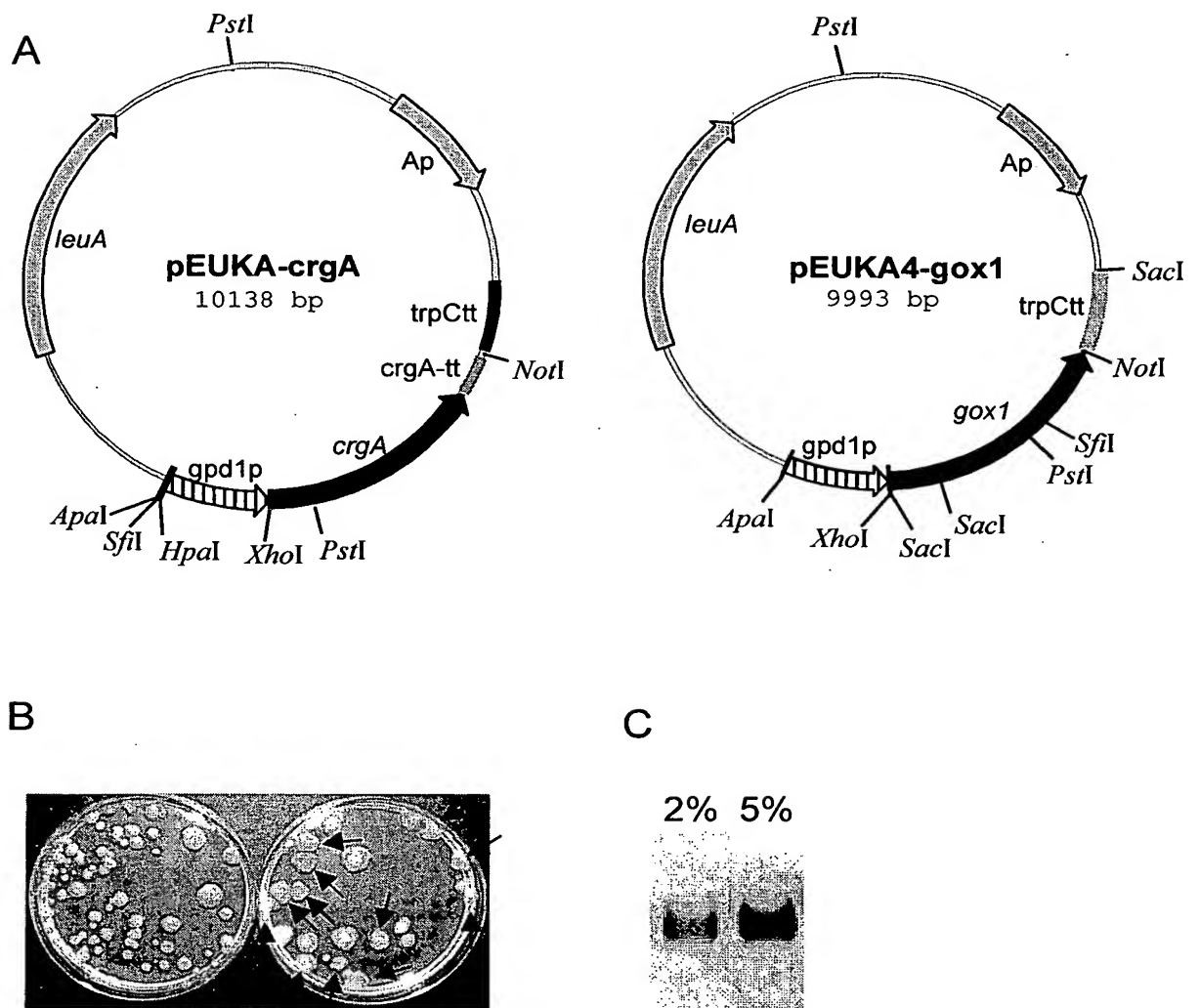


Fig. 18



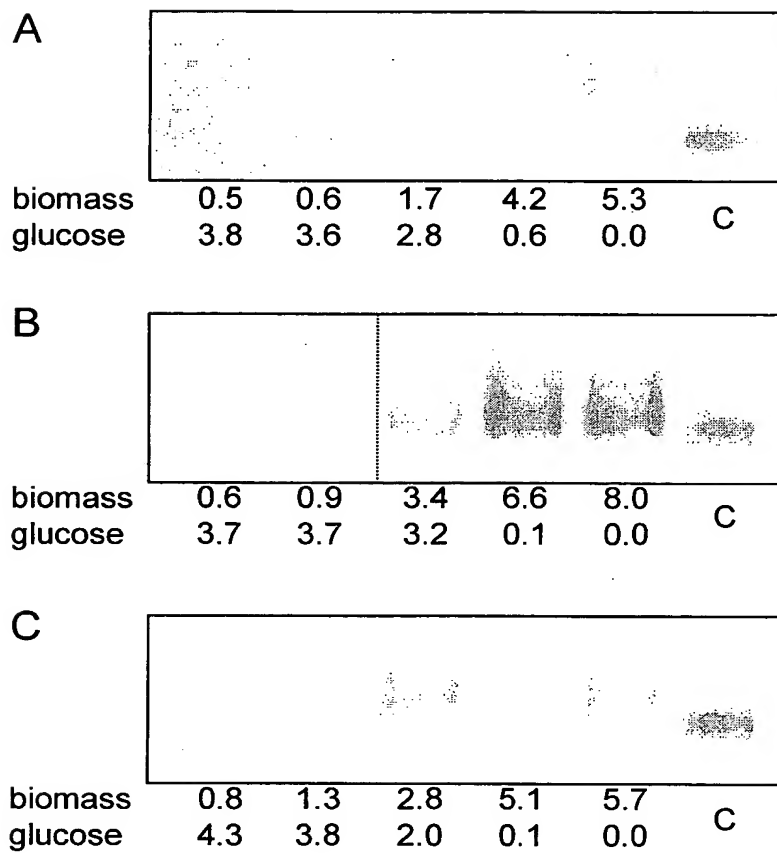


Fig. 19

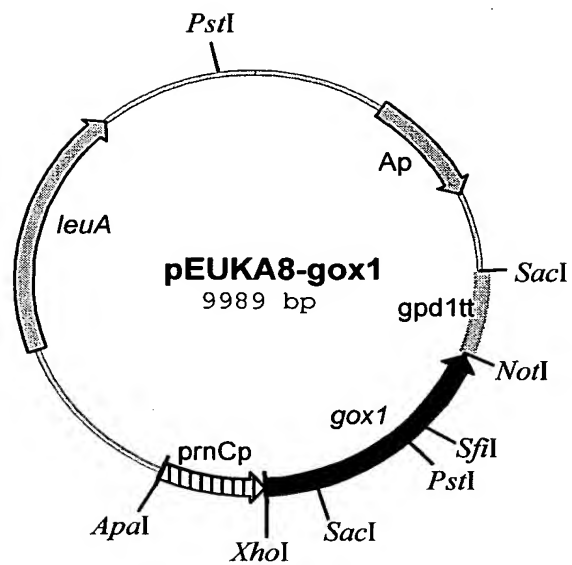


Fig. 20

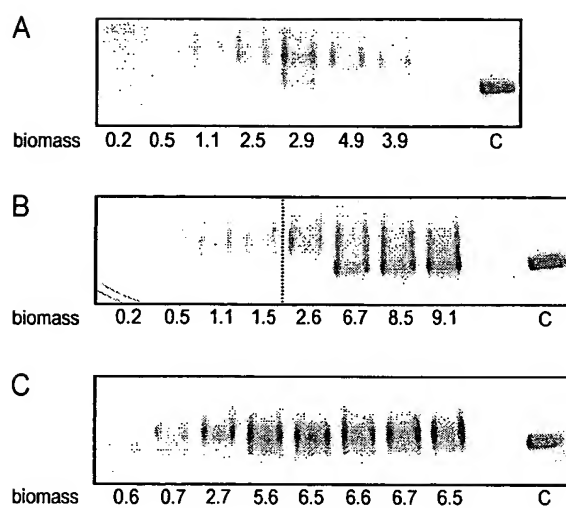


Fig. 21